5-2014

AN ENGINEERING APPROACH TO FORENSIC METHODS: THE CITRATE METHOD FOR POSTMORTEM INTERVAL DETERMINATION

Melissa Dunphy
Clemson University, mdunphy@g.clemson.edu

AN ENGINEERING APPROACH TO FORENSIC METHODS: THE CITRATE METHOD FOR POSTMORTEM INTERVAL DETERMINATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

---

by
Melissa Allyn Dunphy
May 2014

Accepted by:
Melinda Harman, Ph.D., Committee Chair
Elena Mikhailova, Ph.D.
Katherine Weisensee, Ph.D.

ABSTRACT

The Postmortem Interval (PMI) is the amount of time between the death of an individual and the discovery of the remains. Existing forensic techniques for the determination of the PMI are limited once soft tissue fully decays [31, 32]. A novel technique has recently been developed using the molecule Citrate ($C_6H_8O_7$) to calculate the Postmortem Interval (PMI) [26]. Currently, this method cannot be practically applied due to the limited understanding of different forensic environments on the biochemistry of postmortem bone. In addition, further understanding of bone citrate measurement methods is needed to determine a practical field measurement approach. This study aims to (1) identify alternative measurement methods for bone citrate and (2) design a bioreactor system that can discretize the myriad of environmental variables that impact decomposition.

Using a colorimetric assay and high-performance liquid chromatography (HPLC), this study measured bone citrate concentrations at 1.26 and 1.39 wt. % (colorimetric assay measurement) and 1.69 wt. % (HPLC measurement). Compared to the value established by Schwarcz et al. (2013) of 2.0 wt. %, results show 63 %, 69 %, (colorimetric assay measurement) and 85 % (HPLC measurement) extraction and recovery of citrate. However, high standard deviations and intra-sample variance suggest a need for further analysis of the sample preparation methods, as well as assessment of potential techniques for the quantification of citrate concentration.

Forensic bioreactors were designed based on forensically-relevant environmental variables discretized using the following soil science metrics: soil temperature regime,

soil moisture regime, slope, texture, soil horizon, cation exchange capacity, soil pH, and organic matter content. A total of fourteen different environmental conditions were created and controlled successfully over a 60 day experiment. Results demonstrate successful development, implementation and control of forensic bioreactors. A pilot study demonstrates the need for further refinement of chemical methods; however, porcine rib bone samples were successfully aged in bioreactors over 60 days.

This research demonstrates the need for further development of the citrate chemical analysis to ensure accurate field testing. Once a field method for citrate measurement has been established, bioreactor systems can be created to replicate many different of clandestine burial contexts, which will allow for the more rapid understanding of environmental effects on skeletal remains. Ultimately, through the use of forensic bioreactors, global research can be piloted in the local laboratory.

DEDICATION

This thesis is dedicated to Adam Yates, for helping me and supporting me from the drafting of the project idea two years ago to the submission of this thesis. Also, to my family, for supporting me throughout my education.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Melinda Harman, for her help and support (as well as patience) throughout my research at Clemson. It has been an invaluable to work with a research so willing to engage in new disciplines. I also would like to thank my committee, Dr. Elena Mikhailova and Dr. Katherine Weisensee for their guidance and expertise throughout my research. To Dr. Mark Schlautman and Dr. Sarah Harcum, I thank you for all of your chemical expertise during the challenges of my research. Finally, I would like to thank the members, faculty, and staff of RE-MED laboratory, CU-REPRO, CUBEInc., and the Department of Bioengineering, without whose assistance I could not have completed this research.

TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

Figure Page

CHAPTER ONE

PREFACE

This research explores the potential of new methodologies for the advancement of forensic research through the application of bioengineering and soil sciences expertise. Existing techniques in forensics utilize outdoor research facilities to simulate the effects of the environment on bones, and evaluation of new techniques requires testing in many environments to determine the applicability of new methods. This research aims to assess the potential of a new PMI evaluation technique, the citrate method, and to design a system to allow accurate evaluation of environmental parameters on decomposition. Experimentation is organized into two primary phases: the development of chemical methodologies and the development of systems methodologies. Results from this work will provide the framework for further evaluation of the citrate method, as well as a system for laboratory preparation of forensic samples.

In order to progress the development of the citrate method, Chapter 2 considers the existing method for assessing citrate in bone and assess alternative means of determining bone citrate concentration. A colorimetric assay is recommended for consideration and results are compared to high-performance liquid chromatography (HPLC) measurements of forensic bone specimens. Through the consideration of these techniques, the concentration of citrate in bone is determined, and further applied to future research that attempts to use the citrate method.

In order to allow for the accurate determination of the impact of specific environmental factors, Chapter 3 discusses the development of environmental simulators, here defined as forensic bioreactors. Through the combination of anthropology and soil science, environmental variables from forensic taphonomy were discretized to create a system that simulates environmental conditions and ages forensic specimens in a controlled manner. As a result, these systems can be utilized to determine the impact of a specific variable while controlling for other environmental variables. Chapter 4 presents a short-term study conducted over a 60 day period that aims to evaluate the impact of several environmental factors on citrate concentration in bone. Although these results are found to be inconclusive, long term studies conducted in forensic bioreactors and tested using the citrate method may allow for the development of a mathematical model to measure the PMI of samples aged in many different environments.

CHAPTER TWO

ANALYSIS OF A COLORIMETRIC CHEMICAL ASSAY FOR THE MEASUREMENT OF CITRATE IN BONE.

Abstract

Citrate, $C_6H_8O_7$, is a potential indicator of the Postmortem Interval (PMI) for forensic bone samples. Previous research has determined the concentration of citrate in cortical bone to be 2.0 wt. % using a UV enzymatic assay. This study will aim to confirm these results using standard addition methodologies and colorimetric spectroscopy detection to estimate the recovered citrate from bone homogenates. The method facilitates simple and accurate quantification of citrate present in a bone sample, using a colorimetric assay distributed by Abcam, Inc. (Cambridge, MA). Samples were prepared using the gold standard protocol for extraction of citrate from bone. Microplate assay results were compared to values obtained using the same samples tested using high-performance liquid chromatography (HPLC). Citrate concentrations of 1.26 and 1.39 wt. % were obtained using two standard addition methodologies quantified using the colorimetric assay; for the same methodologies, a citrate concentration of 1.69 wt. % was quantified using HPLC. Compared to the value established by Schwarcz et al. of 2.0 wt. %, these results show 63 wt. %, 69 wt. %, and 85 wt. % recovery of citrate respectively using standard addition. However, standard deviations of 0.2 and greater were obtained between six fresh bone samples tested with these methods, and greater variance was found between preparations of the same bone side by side suggesting a need for further

analysis of the sample preparation methods, as well as assessment of potential techniques for the quantification of citrate concentration.

Introduction

In forensic investigations in which human skeletal remains are recovered from a burial site in the outdoor environment, a necessary detail for investigators to determine is the period of time since the person's death, known as the postmortem interval (PMI). A novel method to determine PMI is by measuring the citrate concentration in the recovered bone, which can provide a quantitative estimate of the PMI rather than the more common qualitative approaches [26]. However, most citrate assays have not yet been tested with forensic bone samples, and their ease of use by forensic scientists and accuracy with forensic samples are unknown. This research aims to apply analytical chemistry techniques to bone with known PMI in order to validate a commercially available citrate assay and its use with bone samples prepared using published protocols [17, 26].

Citrate can be used to determine the PMI because it has known expected concentrations in bone and it is believed to have a predictable decomposition rate with time.[1] Bone consists of an organic and inorganic matrix, primarily composed of Type I collagen proteins and calcium hydroxyapatite, respectively. During bone formation, nano-scale collagen fibrils are mineralized by calcium and phosphate binding and embedded with the hydroxyapatite nanocrystals. The inorganic apatite surfaces are regulated by organic matrices, including the molecule citrate, which is bound to approximately $1/6^{th}$ of the apatite surfaces [7, 15, 35]. The organic molecule citrate has

the molecular formula, $C_6H_8O_7$. The role of citrate in bone is linked to normal cell metabolism for adenosine triphosphate (ATP) production in the Krebs Cycle/Citric Acid Cycle and to bone mineralization [7]. Citrate provides a larger number of carboxylates (COO^-) for calcium binding than the other main organic proteins combined, helping to stabilize the hydroxyapatite nanocrystal size and impacting bone strength [15]. The average percent mass of citrate in human bone is 1.6%, which is approximately 20 – 80 μmol/g [13, 26]. With respect to only the organic components of healthy bone, citrate concentration is 5.5% of the organic mass, or about 1.5 to 2 weight percent (wt. %) in bone [7, 13, 26]. Thus, citrate can be considered a useful biochemical marker in bone because it is bound to bone mineral and is maintained at optimal levels through normal cell metabolism and bone remodeling throughout life [7]. When cell metabolism stops at death, the production of citrate halts and the quantity of citrate present in bone becomes finite.

In order to analyze citrate concentration, the molecule must be extracted from the bone tissue after it has been removed from the body or recovered from a burial scene. Demineralization through the use of hydrochloric acid (HCl) reduces the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) in bone into monocalcium phosphate ($CaH_4P_2O_8$) and calcium citrate($Ca_3(C_6H_5O_7)_2$). This reaction is rate limited and has a sharp advancing front; therefore, concentration of the acid, time, and temperature can affect the amount of citrate extracted [12]. Citrate detection also can be suppressed or enhanced by matrix effects [11]. IUPAC defines matrix effects as, "the combined effect of all components of the sample other than the analyte on the measurement of the quantity." If a specific

component can be identified as causing an effect then this is referred to as interference [14, 27]. Using a post-extraction spike method, matrix effects are considered present if the relative standard deviation exceeds 4% [11]. Standard addition tests and calibration curves also are useful for detecting matrix effects [25, 27]. Standard addition assumes that for any given analyte in a solution, there will be an analytical sensor that will respond specifically to that analyte and no other component in the solution. Previous studies of citrate concentration in bone have not reported interference occurs due to coeluting substances, although specific assessment of matrix effects were not discussed [13].

Several different enzymatic assays from different providers have been used to detect citrate in bone. Previous forensic studies used citrate assays from Xygen Diagnostics Inc. (Burgessville, ON) and from R-Biopharm AG (Darmstadt, Germany) to estimate PMI [13, 17, 26]. Other citrate assay kits exist, with variation in properties such as use of enzymatic or colorimetric agents, shelf life, and ability to withstand numerous freeze-refreeze cycles. Kit selection must be based on how long the kit can be stored for use, the associated cost per sample to run, and the ease of use for the forensic scientist.

The purpose of this study is to validate a procedure for measuring citrate concentration in bone. The study aims are: 1) to verify the citrate assay kit for use with porcine rib bones based on standard curve and standard addition curve techniques; 2) to verify the sample preparation method to ensure complete citrate extraction and no significant loss of citrate; and 3) to compare two analytical techniques, namely a colorimetric assay and high-performance liquid chromatography (HPLC).

Materials and Methods

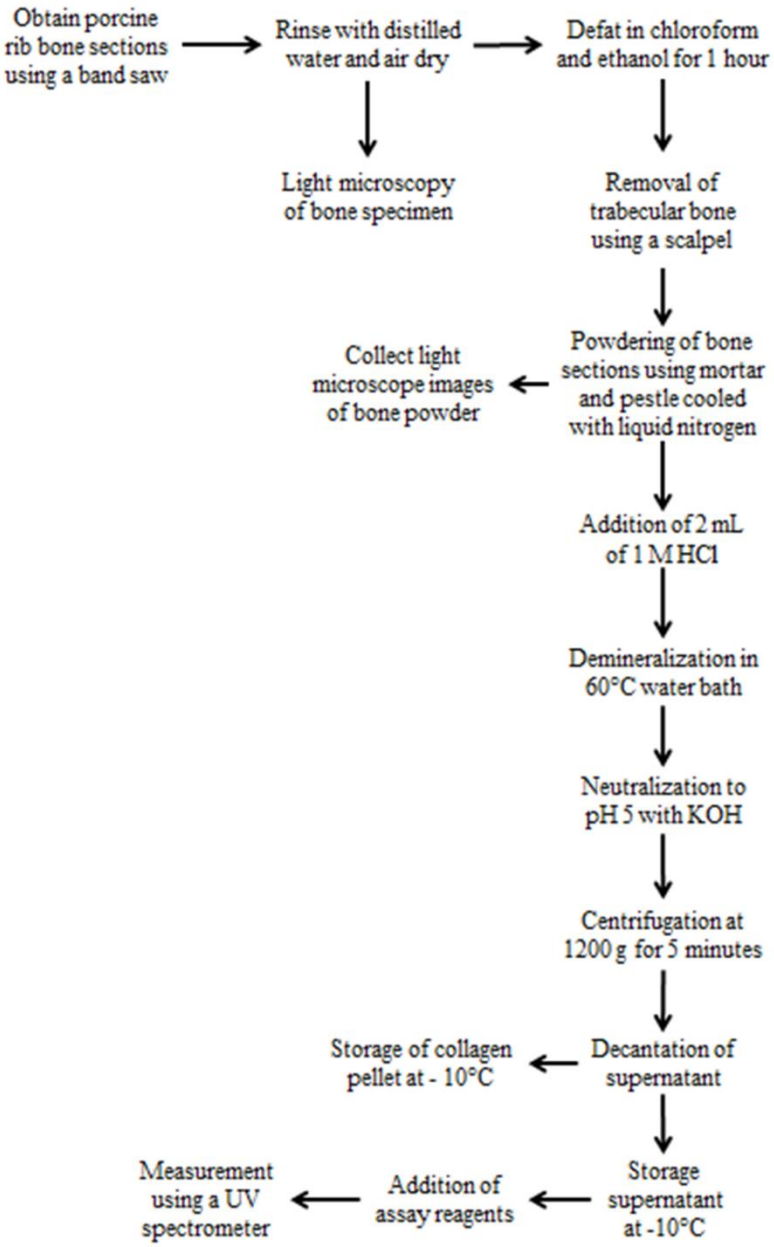
Chemicals

Three commercial citrate assay kits (Kit 1, Kit 2, Kit 3) were obtained (Cat. No. ab83396, Abcam, Cambridge, MA) over a three month period. All components were reagent grade and verified to provide a linear trend for citrate concentrations of 0.1 – 10 nmols per sample. These kits utilize a colorimetric enzymatic method through citrate lyase, in which citrate is converted into oxaloacetate and pyruvate allowing for reaction with the coenzyme NADH. The amount of NAD+ formed in this reaction pathway is stoichiometric with the amount of citrate. For processing of bone specimens, reagent grade chloroform ($CHCl_3$), potassium hydroxide (KOH), sodium hydroxide, laboratory grade ethanol (C_2H_6O), and hydrochloric acid (HCl) were obtained from Fisher Scientific. Sodium citrate dihydrate ($C_6H_9Na_3O_9$) also was obtained from Fisher Scientific to be used in standard addition testing and creation of citrate standards. Nanopure deionized water (Milli-Q ZMQSP0D01, Millipore S.A.S, France) was utilized during all experimental stages.

Bone Solution Preparation

Similar to the previous study by Schwarcz, et al., fresh-frozen porcine spare rib bones were obtained from a local grocer, defleshed and stored at -10°C until being used for bone sample preparation (Figure 2.1) [26]. Individual rib bones were cut into 2-4 mm sections using a band saw and cleaned using a room temperature bath of deionized water.

Figure 2.1: Procedure for the preparation of porcine rib bone citrate solutions. Procedure for the preparation of porcine rib bone specimens based on protocols by Schwarcz et al. and Kanz et al.[1, 15]



Samples were then placed in a mixture of chloroform and ethanol for one hour to remove fatty substances that would interfere with the assay and rinsed again using deionized water. Trabecular bone was removed from cortical bone using a scalpel and then all bones were allowed to air dry for 30 minutes. Separate bone powders of cortical and trabecular bone were produced using a mortar and pestle cooled with liquid nitrogen. Particle size was verified to be less than 10 µm using optical light microscopy (model K400P, Motic, Inc., Xiamen, China) and digital imaging (Infinity 2, Lumenera Corp., Ottawa, Ontario, Canada). Hydroxyapatite was dissolved to liberate the citrate into solution by placing two 50 mg aliquots of bone powder in 15 mL centrifuge tubes, adding 2 mL of 1 M HCl to each, and then placing the tubes in a hot water bath at 60°C for 1 hour. The tubes were removed and the samples neutralized by adding dropwise 0.5 M KOH until a pH of 5 was obtained. Samples were then centrifuged at 1200 g for 5 minutes to reduce collagen to a pellet. The clear supernatant (hereafter, Bone Solution) was decanted into new 15 mL centrifuge tubes and then both pellet and supernatant were stored at -10°C until use. .

Assay measurement

Total citrate concentration in solutions was analyzed using ultraviolet (UV) spectroscopy (Epoch microplate, BioTek, Winooski, VT) at 570 nm wavelength and high-performance liquid chromatography (HPLC) (Dionex, Thermo Fisher Scientific Inc.). For UV spectroscopy, the Bone Solution was plated in 1-5 µl volumes into 96 flat-bottomed well microplates and appropriate quantities of kit buffer and chemical reagents were added. For

HPLC, the Bone Solutions were brought to a pH of 2 and filtered by an external laboratory and measured using in an autoanalyzer overnight.

Generation of citrate standard curves

Standard curves were generated using the 1 nmol/µL citrate standard provided in the three kits (Kit Standard), as well as a 1 nmol/µL standard solution was created by adding 15 mL nanopure water to 7.7 mg of granular sodium citrate dihydrate (Lab Standard). These standard solutions were added in volumes of 0 to 10 µL in 2 µL intervals for a total of 6 wells and brought to a final volume of 50 µL using the buffer solution (Table 2.1). These volumes were selected to provide for citrate concentrations of 0 – 10 nmol, or a detection range of 0.0189 to 1.891 mg/mL citrate. As Standard curves for the Kit Standard were measured using HPLC by the external laboratory, as previously described.

Verification of assay to detect citrate concentrations extracted from bone (Table 2.1)

The bone sample preparation method was verified using a standard addition test and citrate retention test to ensure it produced no significant loss of citrate. Volumes of 2 µL of Bone Solution were added to five microplate wells and then 0 to 4 µL of Lab Solution were added in 1 µL intervals and each well brought to 50 µL using buffer. The citrate retention test was used to evaluate the potential for the undesired digestion of

Table 2.1: Procedure for the evaluation of the citrate assay with porcine bone specimens. Procedures for the evaluation of the citrate assay with porcine bone specimens. The standard curve test validates that the assay is in proper condition. The standard addition test recognition of potential matrix effects, if any, by the addition of a standard solution to an unknown sample. The citrate retention test evaluates potential for digestion of citrate during sample processing at the step where citrate is released into solution.

Protocol	96 Well Plate Cell					
	1	2	3	4	5	6
Standard curve						
Sample [µL]	0	0	0	0	0	0
Standard [µL]	0	2	4	6	8	10
Buffer [µL]	50	48	46	44	42	40
Standard addition						
Post extraction spike method						
Sample [µL]	2	2	2	2	2	
Standard [µL]	0	1	2	3	4	
Buffer [µL]	48	47	46	45	44	
Citrate retention						
Sample [µL]	1	1	1	1	1	
Sodium Citrate [mg]	0	1	2	3	4	
Buffer [µL]	49	49	49	49	49	

citrate during sample processing at the bone demineralization step where citrate is released into solution (60°C water bath while in 1 M HCl for 1 hour). Bone powder was prepared as previously described (Figure 2.1) up to the step before bone demineralization. During the aliquoting step for creating 50 mg bone powder samples, sodium citrate dihydrate was added in 0 to 4 mg quantities in 1 mg intervals for a total of 5 samples. Each sample was then carried through the remaining steps of the sample processing procedure. The expected change in citrate concentration was 0.114 mg/mL based on addition of 1 milligram of citrate to an average sample volume of 5.7 mL. Once processed, 1 µL of sample was added to each of five microplate wells and brought to a final volume of 50 µL using buffer. For both the standard addition test and citrate retention test, kit reagents were added according to the kit protocol and samples were analyzed using the UV spectrometer. Volumes of the same samples were sent to the external laboratory for HPLC analysis, as previously described.

Replication

Three bone sections were cut from whole porcine rib bones, prepared into powder as previously described (Figure 2.1), and measured into 50 mg aliquots in duplicates. Bone solution was collected from individual supernatant samples were then assayed in duplicate. All remaining whole bone, bone sections and bone powder samples were stored at -10°C until the end of this experiment

Results

Standard curve assessment of bone citrate concentration

Using the Kit Standard and Lab Standard solutions, standard curves were linear over the established range of 0.0189 to 1.891 mg/mL when measured using the colorimetric assay (Figure 2.1). Replication of standard curves using the Kit Standard from three separate citrate assay kits showed consistent response linearity ($R^2 = 0.9967$) (Figure 2.2). Using the line of best fit for standard curve data that was modified to solve for wt. % (Equation 1), citrate concentration of a given Bone Solution (CA_o) was calculated from the measured optical density (OD).

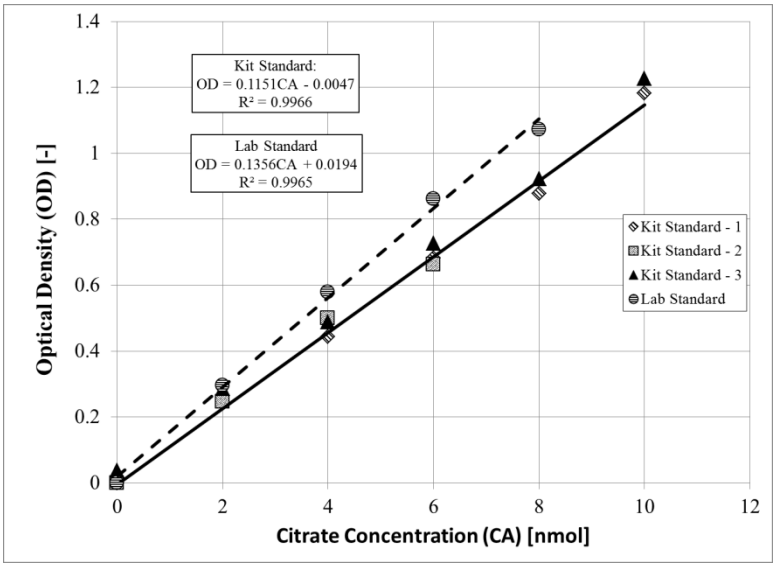
$$CA_o = \frac{OD - 0.0047}{0.115} * \frac{9.6}{C_{bone}} \text{ [wt. \%]} \quad (1)$$

The value 9.6 is a conversion constant calculated using the molar mass of citrate and basic unit conversions. The bone concentration of the Bone Solution (C_{bone}) was calculated based on the mass of bone added (m_{bone}), for this experiment 50 mg, and total volume of HCl and KOH added during processing (V_s) using Equation 2.

$$C_{bone} = \frac{m_{bone}}{V_s} \text{ [mg/mL]} \quad (2)$$

Using Equations 1 and 2, the citrate concentrations of two sets of porcine rib bone samples were calculated. Set 1 Bone Solutions included six homogenate samples prepared with cortical and trabecular bone powder. Set 2 Bone Solutions included three homogenate samples prepared with cortical bone powder. The mean citrate concentration of was 1.01 ± 0.18 wt. % for Set 1 and 1.27 ± 0.24 wt. % for Set 2 (Table 2.2). Additional

Figure 2.2 Standard curves for citrate concentration calculation. Citrate standard curve created using Kit Standard and Lab Standard measured with the colorimetric assay and HPLC. . The linear trends were used to calculate the unknown citrate concentration in the Bone Solutions. Error is shown between samples obtained using Kit Standard for all three kits. A sample size of n=3 for concentrations of 0, 2, 4, 6 nmol, and n=2 for concentrations of 8, 10 nmol were used. Due to the desired testing range for porcine rib bone, fewer samples were analyzed at higher concentrations. Equation 1 was created using the line of best fit for Kit Standards.



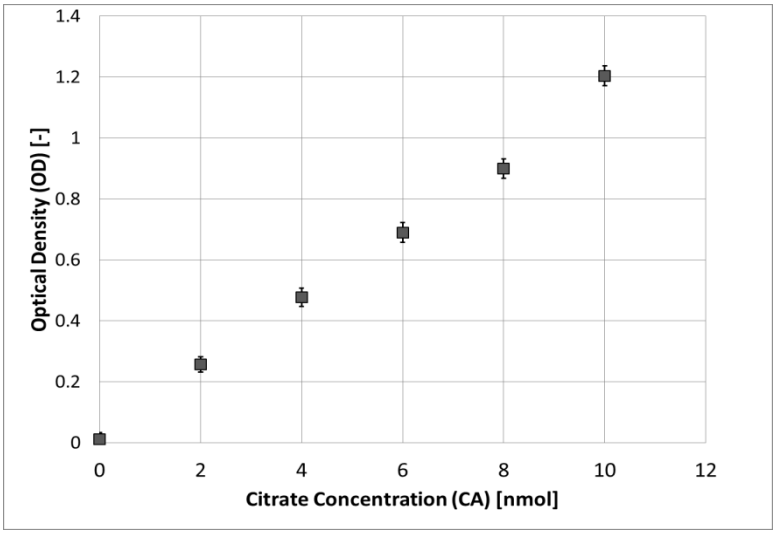


Table 2.2: Citrate concentrations measured with colorimetric assay. Citrate concentrations in Bone Solutions measured using the colorimetric assay. Samples include cortical and trabecular bone combined into the bone powder, and cortical bone only included in the bone powder.

Porcine Rib Bone Citrate Concentration Calculations		
Set Number	Optical Density (OD) [-]	Citrate Concentration (CA_o) [wt. %]
Set 1: Cortical and Trabecular Bone Solutions		
Bone 1	0.347	1.16
Bone 2	0.282	0.93
Bone 3	0.379	1.16
Bone 4	0.256	0.79
Set 2: Cortical Bone Solutions		
Bone 5	0.188	1.52
Bone 6	0.157	1.25
Bone 6	0.316	1.05

cortical bone powder samples were measured using HPLC (Set 3 Bone Solutions), resulting in a mean citrate concentration of 0.94 ± 0.22 wt. % (Table 2.3).

Verification of assay to detect citrate concentrations extracted from bone

The standard addition curve created by incremental addition of Kit Standard to Bone Solution was linear (R^2 = 0.9965) over a range of 0 to 4 nmol when measured using a colorimetric assay (Figure 2.3). The unknown citrate concentration of the Bone Solution (CA_o) was determined using the line of best fit for these data, modified into Equation 3. Using Equation 3, and the bone concentration in solution, C_s = 9.615 mg/mL, the citrate concentration of Bone Solution was calculated to be 1.26 wt. %.

$$CA_o = \frac{0.114}{0.0899} * \frac{9.6}{C_{bone}} \text{ [wt. \%]} \quad (3)$$

The standard addition curve created by incremental addition of granular sodium citrate dihydrate in to bone powder prior to demineralization (citrate retention curve) was linear (R^2 = 0.9798) over a range of 0 to 4 mg when measured using a colorimetric assay (Figure 2.4). The unknown citrate concentration of Bone Solution (CA_o) was determined using the line of best fit from Figure 4, modified into Equation 4. Using Equation 4 and Bone Solution bone concentration, C_s = 9.615 mg/mL, Bone Solution citrate concentration was calculated to be 1.39 wt. %.

$$CA_o = \frac{0.0324}{0.1205} * \frac{50}{C_{bone}} \text{ [wt. \%]} \quad (4)$$

The value 50 is a conversion constant calculated based on the molar mass of citrate and basic unit conversions.

Table 2.3: Citrate concentrations measured with colorimetric assay. Citrate concentrations for Bone Solutions measured using HPLC**.** The computed mean citrate concentration is 0.94 $\pm$ 0.22 wt. %.

Citrate concentration (Ca_o) [wt. %] obtained using HPLC		
Set Number	Run Number	
Set 3: HPLC Bone	Run 1 [wt. %]	Run 2 [wt. %]
Bone 1	1.07	1.19
Bone 2	1.10	0.76
Bone 3	0.67	0.95
Bone 4	0.89	0.89
Bone 5	0.49	0.98
Bone 6	1.21	0.87
Bone 7	1.08	0.67

Figure 2.3: Standard addition curve by addition of volume measured with colorimetric assay. Standard addition curve generated using the colorimetric assay and spectroscopy for assessing matrix effects on the Bone Solutions. The resulting line of best fit is used in Equation 3 to calculate an initial citrate concentration of 1.26 wt. %

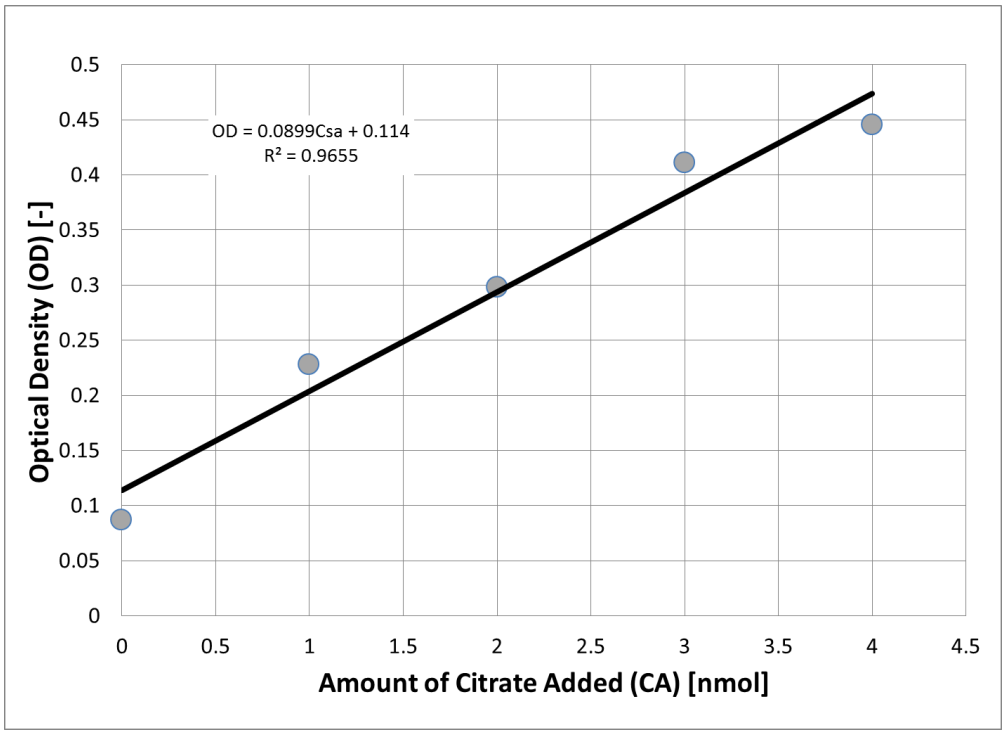
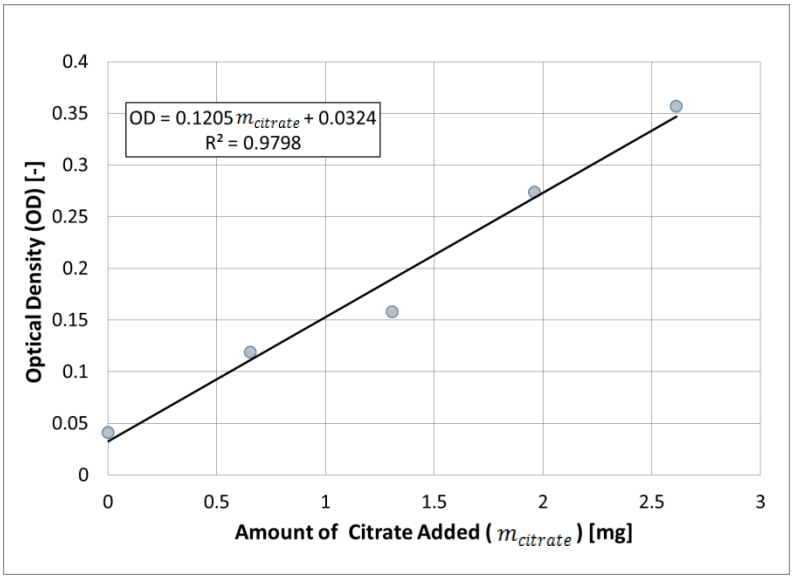
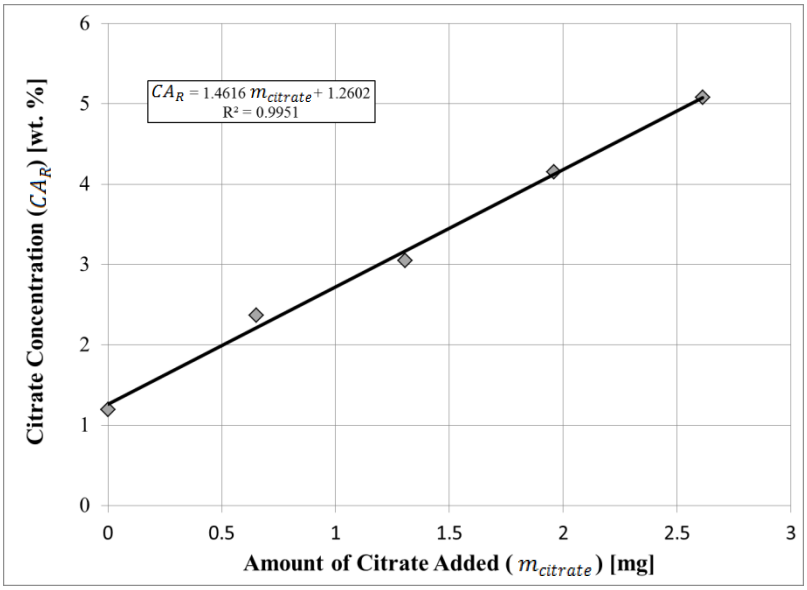


Figure 2.4: Standard addition curve by addition of granular citrate measured with colorimetric assay and HPLC respectively. Citrate retention tests conducted using a colorimetric assay and HPLC to determine if citrate is lost during bone demineralization and the release of citrate into solution using a 60°C HCl acid bath for 1 hour. This resulting line of best fit equations are used in Equations 4 and 5 to calculate an initial citrate concentration of 1.39 wt. % and 1.69 wt. % for the colormetric assay and HPLC, respectively.





These samples were then analyzed using HPLC. The data were linear (R^2 = 0.9951) over a range of 0 to 4 mg (Figure 2.4). The unknown concentration of the Bone Solution (CA_o) was determined using the line of best fit for this data, modified into Equation 5. Using Equation 5, citrate concentration of Bone Solution was calculated to be 1.69 wt. %.

$$CA_o = \frac{1.260}{1.462} \text{ [wt. \%]} \quad (5)$$

Assessment of citrate concentration retention in bone solutions

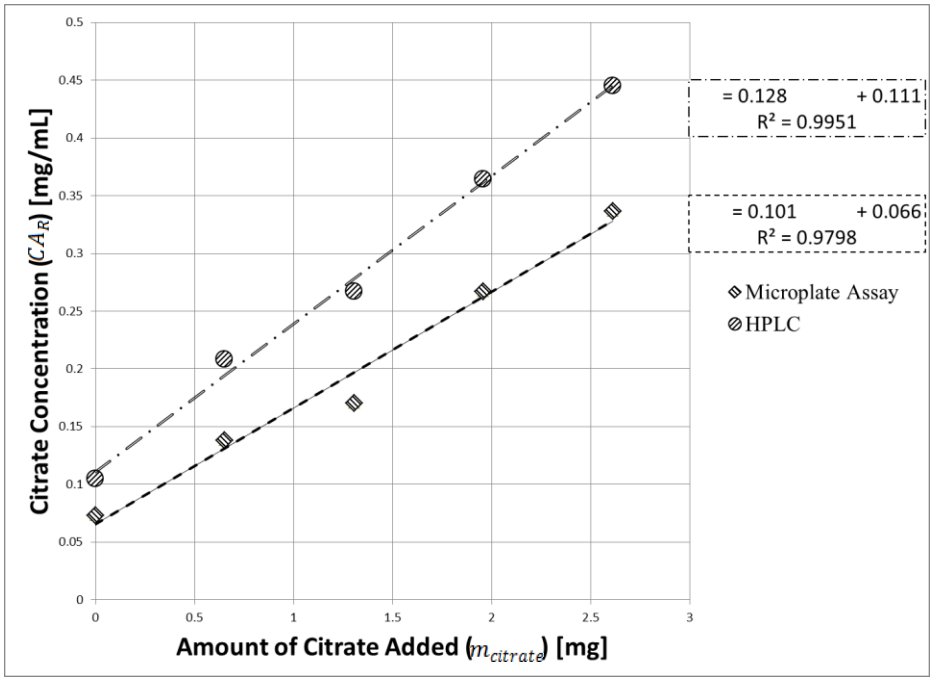
Citrate retention curves were converted to show Citrate Concentration [wt. %] on the ordinate versus mass of added citrate on the abscissa (Figure 2.5). Using the line of best fit for the assay data, Equations 6 and 7 calculate citrate concentration in wt. % (CA_R) based on the amount of added citrate ($m_{citrate}$). These equations were used to determine the unknown change in Bone Solution citrate concentration based on the addition of 1 mg of sodium citrate dihydrate.

$$CA_R = 0.101 m_{citrate} + 0.066 \quad (6)$$

$$CA_R = 0.128 m_{citrate} + 0.111 \quad (7)$$

The slope factor in Equation 6 determines a 0.101 mg/mL concentration increase per milligram of citrate added based on measurement of citrate concentration using the colorimetric assay and colorimetric spectroscopy. The slope of Equation 7 determines a 0.128 mg/mL concentration increase per milligram of citrate added based on measurement of citrate concentration using HPLC.

Figure 2.5: Comparison of colorimetric assay and HPLC results of citrate retention test. Data from the citrate retention tests using the different analytical techniques. Citrate concentrations obtained using the colorimetric citrate assay demonstrate a concentration increase of 0.101 mg/mL per milligram of citrate added to the bone powder aliquot. In comparison, citrate concentrations obtained using HPLC demonstrate a concentration increase of 0.128 mg/mL per milligram of citrate added to the bone powder aliquot.



Discussion

This study sought to validate a procedure for measuring citrate concentration in bone. The colorimetric citrate assay kits were verified for use with porcine rib bones using standard curve and standard addition curve techniques. The sample preparation method was not validated, as complete citrate extraction and no significant loss of citrate were not confirmed. The colorimetric spectroscopy and HPLC were repeatable over multiple runs of the same Bone Solution, but there are noted differences in measured citrate concentrations for the same samples.

Both colorimetric spectroscopy and HPLC proved useful for detecting citrate in Bone Solutions produced from porcine rib bones. These data verified the colorimetric citrate assay kit can generate standard curves with sufficient linearity ($R^2 > 0.99$, Figure 3) for calculating citrate concentration from optical density when detected using colorimetric spectroscopy and a microplate assay. In addition, linear standard addition curves were possible using both the colorimetric assay and the HPLC techniques (Figure 2.3 and Figure 2.4).

This study sought to validate a procedure for measuring citrate concentration in bone. Bone samples were collected and processed using the protocols reported by Kanz et al. and Schwarcz et al [17, 26]. Some modest changes were made, including the addition of optical microscopy and imaging to confirm relatively uniform particle sizes were achieved in the bone powdering step, storage of the collagen pellet, and use of a colorimetric microplate assay instead of a colorimetric microplate assay. Total time required from rinsing bone sections through centrifugation required approximately 2.5 hours with an addition 0.75 hours required for micro-pipetting and colorimetric

spectroscopy. The final procedure provided additional confirmation steps for the testing of citrate concentration in bone (Figure 2.1).

In order for measurement of bone citrate concentration to be a viable option for forensic anthropology, the analytical techniques must be easily executed and rigorously proven to generate accurate results with forensic bone samples. In the current study, the citrate assay kits and colorimetric spectroscopy techniques were verified using porcine rib bones and analytical chemistry and then compared to HPLC. Standard addition curves demonstrated linearity of measured parameters (Figure 2.3 and Figure 2.4).Using the colorimetric assay, initial concentration of the Bone Solution was 1.26 wt. % for the standard addition of the Lab Standard solution and 1.39 wt. % for the standard addition of granular Sodium citrate dihydrate to the bone powder during sample preparation. When measured using HPLC, the initial concentration of the Bone Solution was 1.69 wt. % based on the standard addition of granular Sodium citrate dihydrate to the bone powder.

Previous studies report citrate concentrations in trabecular and cortical bone are approximately 1.5 wt. % and 2 wt. %, Gibbs and Schwarcz et al. respectively [13, 26]. Bone citrate concentrations measured in the current study are lower than these expected values; however, it is necessary to identify whether citrate is lost during processing or if the selected assay is not specific enough to detect all citrate that is present in solution. Bone sample preparation methods were followed as specified in Kanz et al. and Schwarcz et al [17, 26]. It was implied, though not explicitly stated in Schwarcz et al., that only cortical bone was selected for bone citrate sample preparation. Using commercially purified bovine bone mineral (NuOss™, Collagen Matrix Inc.) and nuclear magnetic

resonance, Hu, et al. measured 0.8 mg of citrate per 190 mg of bone mineral [15]. Further studies utilizing such commercially available bone with known amounts of citrate are needed to fully verify the analytical techniques utilized.

Using data extrapolation to calculate initial concentration of Bone Solutions, it was determined that citrate loss or interference was occurring. Based on retention tests, there is potential for citrate loss up to at least 12.3 % between sample preparation and testing the sample with the assay. However, this does not account for all loss seen in samples calculated in Tables 2.2 and 2.3, suggesting the presence of matrix effects or other interference, for example calcium binding at enzymatic pH levels. Therefore, further testing is needed to assess potential causes of interference. Potential tests could consider a method that tests at a lower pH or that can limit the presence of calcium in solution.

Assuming an initial citrate concentration in porcine rib bone of 2 wt. %, as reported by Schwarcz, et al [26]. there was less than 70% citrate recovery using standard addition methods and measurement with the colorimetric assay and approximately 85% recovery using HPLC measurement. Based on these differences, as well as the results of the citrate retention test, the amount of citrate present may be less than 2.0 wt. % in porcine cortical bone samples. For Bone Solutions run in duplicate using both the colorimetric assay and HPLC techniques, inter-sample variation ranged from 0.18 to 0.24 for both techniques. In addition, Bone Solutions prepared from the same bone powder in identical conditions had deviations of 0.1 – 0.4 wt. %, suggesting a need for refinement in sample preparation methods to ensure precise PMI calculations using these techniques.

Conclusion

In order to develop an analytical technique for assessing PMI based on bone biochemistry, it is necessary to further refine the sample preparation procedures to ensure reproducibility between forensic labs. The existing protocol has been refined in this study to improve repeatability, and colorimetric assay and HPLC techniques have been tested on these samples prepared using this protocol. The variation in measured citrate concentration between the colorimetric assay and HPLC techniques suggest additional work is needed to determine the cause of variation. In addition, further testing of the protocol used by Schwarcz et al. and Kanz et al. is needed to determine if citrate is lost due to matrix effects, citrate loss, or errors in established values [17, 26]. Higher citrate concentrations may be possible to achieve if demineralization times are extended, increased powdering of bone sections is conducted, and measurement at more acidic pH values. Ultimately, this research demonstrates the potential of colorimetric assay techniques for measuring citrate concentration in bone. Additional work is needed before the bone preparation technique and analytical chemistry techniques can be recommended for forensic fieldwork.

CHAPTER THREE

DESIGN AND EVALUATION OF BIOREACTORS TO MODEL FORENSIC BURIAL ENVIRONMENTS.

Abstract

Existing forensic taphonomic methods lack specificity in determining the postmortem interval (PMI) in the period following active decomposition. New methods, such as the use of citrate concentration in bone, are currently being considered; however, determining the applicability of these methods in differing environmental contexts is challenging. This research aims to design a forensic bioreactor that can account for environmental factors known to impact decomposition, specifically temperature, moisture, physical damage from animals, burial depth, soil pH, and organic matter content. These forensically-relevant environmental variables were characterized in a soil science context. The resulting metrics were soil temperature regime, soil moisture regime, slope, texture, soil horizon, cation exchange capacity, soil pH, and organic matter content. Bioreactor chambers were constructed using sterilized thin-walled polystyrene boxes housed in calibrated temperature chambers. Gravesoil organic matter content was determined using mineral soil (Ultisols), and organic soil proxy for Histisols, horticulture mix. Gravesoil depth was determined using mineral soil horizons A and Bt2 to simulate surface scatter and shallow grave burial respectively. A total of fourteen different environmental conditions were created and controlled successfully over a 90 day experiment. These results demonstrate successful implementation and control of forensic

bioreactors simulating precise environments in a single research location, rather than testing on site. Using this methodology, bioreactor systems can be created to replicate many different of clandestine burial contexts, which will allow for the more rapid understanding of environmental effects on skeletal remains.

Introduction

Accurate estimation of the Postmortem Interval (PMI) provides context to forensic investigations. The PMI defines the time elapsed between death and discovery of human remains [31]. Methods for PMI evaluation are well established for recovered remains with soft tissues, which can be present up to 1285 average degree days (ADD), or a PMI of approximately six months or less; however, precise methods for PMI evaluation of skeletal remains continue to elude researchers [17, 26, 31, 26]. Change in citrate concentration is a novel method being developed in forensic research that utilizes the biochemistry of bone specimens to determine the PMI [17, 26]. However, the impact of the burial environment on the change in citrate concentration over time is not well understood [17]. Without an understanding of these factors, this method cannot be readily applied to regions with different environmental conditions.

Decomposition is categorized into five phases based on the physicochemical and bacterial environments: autolysis, putrefaction, liquefaction, dissolution, and skeletonization [9]. Soft tissues break down due to microbial decomposition and natural cell death occurs during the first four phases, while bone diagenesis occurs due to mechanical breaking, decalcification, and dissolution in acidic soils or water during

dissolution and skeletonization phases. Taphonomy aims to understand a decomposition site through the study of ecology, identifies environmental factors that can lead to body decomposition after death [28]. The primary taphonomic factors include: water movement, temperature regime, organic matter content, fluoride and carbonate concentration, duration of internment in soil, soil pH, cryoturbation, microbial activity, release of fat, and mineralogy [23, 29]. Using a mechanistic approach to understand the distinct variables in this complex system, these taphonomic factors can be systematically controlled in a forensic bioreactor to empirically determine their impact on bone biochemistry.

Bioreactors allow for the reproduction and control of specific environments with the goal of understanding biological, chemical, or physical processes through the manipulation of pH, temperature, oxygen content, mechanical tension, and nutrient [24]. Carter et al. (2008) successfully implemented a forensic temperature incubation system over 28 days for the assessment of rat cadaver decomposition with respect to temperature (15 - 29°C) and microbial activity [3]. In a shorter term study, Abdul-Maksoud (2010) implemented an archeological temperature incubation system over 13 hours for the assessment of bone specimens with respect to temperature (200 - 300°C) [3]. Both of these studies attempted to identify an environmental mechanism for the changes seen in cadaver specimens [10, 11]. Carter et al. (2008) found that temperature acted as an environmental mechanism and increased microbial activity, which is known to impact decomposition [3]. Abdul-Maksoud (2010) found that temperature as an environmental mechanism impacted coloration and crystallinity in such a way that artificially aged bone

samples could be matched to archeological bone samples [1]. Based on the results of these studies, the simulation of other environmental mechanisms to evaluate their impact on decomposition, would aid in understanding how cadaveric samples progress in burial conditions over time. Through the design of a bioreactor system capable of controlling existing parameters known to impact decomposition, many different burial scenarios could be considered side by side.

Design parameters for existing bioreactor systems are well described, thus a system can be designed to incorporate discrete variables relevant to forensic taphonmy. Common design parameters include: size of the apparatus, temperature, sterility, oxygenation, ability to apply sensors, use over time, transparency of the bioreactor, easy placement of the sample, and easy replacement of the medium [19]. Given the chemical properties that soil imparts to the system, soil becomes the medium for this bioreactor [23]. Similarly, as microbial activity is a hallmark of decomposition processes, the bioreactor can be used for its intended purpose – the cultivation of microbial populations [24]. In evaluation of the apparatus, chamber type, sample type and sample size must be considered.

The purpose of the current study is to design and develop a forensic bioreactor as a method for controlling key environmental variables affecting bone decomposition, specifically temperature and soil, in order to systematically evaluate their impact on bone biochemistry over time. A synergistic collaboration between relevant disciplines is pursued, including bioengineering to define the biosystems control of the bioreactor, forensic anthropology to define factors impacting bone decomposition, and soil science to

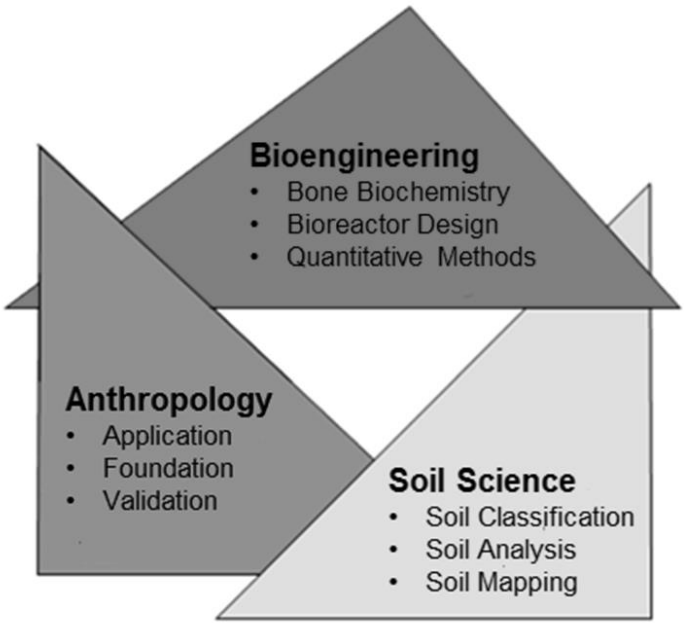
define the soil types and related environmental variables (Figure 3.1). The specific objectives are: 1) to identify the environmental factors most important for modeling burial settings in an outdoor context that are useful for forensic anthropology; 2) to discretize environmental factors of an outdoor forensic burial setting using soil science data; and 3) to design a forensic bioreactor that can be easily monitored to mimic an outdoor burial setting.

Materials and Methods

Defining a baseline of environmental factors through forensic taphonmy

Human remains in clandestine burials are interned in gravesoils. These soils define the environments that forensic specimens are exposed to during decomposition and lead to the acceleration or deceleration of natural decomposition processes [5, 9, 31]. The factors contributing to body decomposition after death can be divided into several categories, including physical (temperature, water movement, animal factors), chemical (mineral content, pH, dissolved gases), microbial (microbial activity, organic matter content), and internment time. Many of these factors are related to the composition of the soil and can be categorized into discrete types using soil science taxonomies. Using a subset of these environmental descriptions, Vass et al. proposed a mathematical model for predicting PMI that focuses on temperature, moisture, pH, and partial pressure of oxygen [31].

Figure 3.1: Identification of synergy between collaborating fields: Anthropology, Bioengineering, and Soil Science.



Identification of measurable environmental factors in forensic taphonmy

Temperature is the most well studied physical factor associated with the rate of decomposition and PMI research [1, 3, 31]. Specifically, temperature leads to an increase in microbial activity resulting in accelerated decomposition during the first four stages [3]. It can also lead to evaporation of available moisture in a given soil environment, thus decreasing microbial activity. Considering both temperature and moisture together, arid environments can lead to mummification, while tropical environments can lead to skeletonization [4, 29]. In the case of skeletonized remains, temperature also leads to measurable alterations in bone histological morphology [5]. Temperature varies depending on region and elevation and can be measured using instantaneous or continuous temperature probes [28, 30]. Temperature variations over time are considered in accumulated degree days, whereby the mean daily temperature is summed over the entire duration of exposure. Soft tissue decomposition, and thus the onset of skeletonization, is considered to occur after 1285 accumulated degree days [32].

Water movement and moisture content is also directly associated with rate of decomposition [2, 4, 17]. Decomposition accelerates in wetter environments, is slowed in lower moisture environments when activity and motility of microbial populations will be restricted; and is halted entirely in excess moisture environments that stop microbial activity [4]. Another way that moisture changes decomposition rates is through the formation of pores in bone that allow exposure to microbial activity [9]. For skeletonized remains, moisture can lead to cracking or splitting of the bone [17]. Moisture varies dependent on region and elevation and its effect on soil water movement and soil

moisture can be evaluated using soil moisture regimes [28]. Alternately, it can be directly measured locally using a soil moisture probe or dry and wet weight comparisons [28].

Disruption by animals can physically alter the exposure of human remains through processes such as trampling, gnawing, and digestion [29]. Increased exposure of hard and soft cadaver tissues to environmental temperature, water movement, and microbial activity accelerates decomposition [2]. These variables are not directly relatable to soil science metrics; however, they can be accounted for during sample preparation.

Considering chemical factors, the gravesoil present at a burial site defines a cadaver's chemical environment. The minerals commonly found in soil that can influence decomposition include: Calcium, Magnesium, Sodium, Potassium, Silicon, Aluminum, and Iron [23]. These minerals, specifically the cation Ca^{2+}, can compete with hydrogen ions found in the soil for sites on hydroxyapatite during the decomposition process and lead to increased or decreased mineral phase dissolution of bone [34].

The pH of soil measures the presence of protons. Soil pH influences the solubility of hydroxyapatite, the primary inorganic component of bone [33, 34]. As soil drops below a pH of 6, hydroxyapatite can be reduced more quickly and release calcium and phosphorous ions. If pH becomes alkaline, reduced rates of dissolution are seen [23]. In addition, pH influences the activity of microorganisms, such as fungi or bacteria [14]. The pH of soil can be measured using soil analysis or a soil pH probe in the field.

Dissolved gasses impact the type of microbial decomposition present. Oxygen levels are increased in soil when a burial site is formed due to aeration of soil [9]. Depending on final grave depth, clandestine burials can be aerobic with primary

decomposition occurring through proteolysis or anaerobic with bacterial fermentation as the primary decomposition mechanism [9, 31]. These levels can be estimated based on presence of soils from different horizons in the burial to estimate initial aeration of gravesoils, as well as through laboratory testing.

Microorganisms are found in soil organic matter, which consists of metabolic byproducts from decomposition as well as soil humates [5]. Microbial decomposition augments the autolysis phase of decomposition, which in turn increases the amount of soil organic matter present in a gravesoil. Although bacteria, microflora, and fungi metabolism are known to impact the postmortem microstructure, research suggested that some additional cofactor must exist to account for the variation seen in the postmortem intervals [2]. Temperature, moisture, aeration and soil type directly impact the rate of decomposition through these processes by altering amount of microorganisms and metabolism of these organisms [3, 4, 5, 9, 29]. The results of this investigation are summarized in Table 3.1.

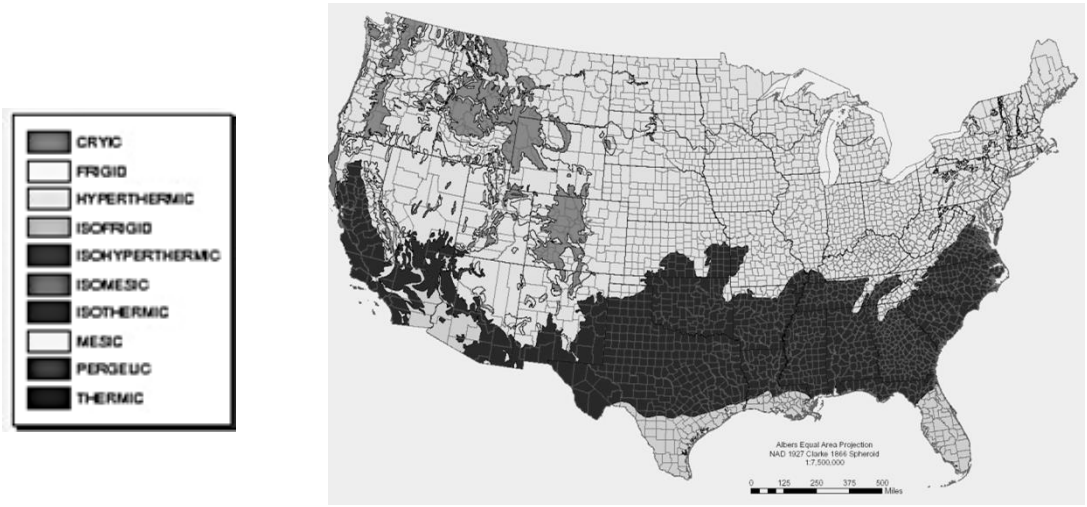
Discretization of bioreactor inputs based on environmental factors.

Soil temperatures can be discretized based on the soil temperature regime for defined geographic regions. Soil temperature regimes have been mapped to allow visual assessment for a region (Figure 3.2). These regimes consider the mean annual temperatures, as well as the comparison between mean summer and mean winter temperature, at a soil depth of 50 cm [30]. Soil temperature maps can be obtained for many global locations.

Table 3.1: Identification of forensic taphonomy variables and associated soil science metrics. Taphonomic factors describe the physical, chemical, and microbial factors identified to impact decomposition. The corresponding soil science metrics describe methods of discretization using existing experimental methods in soil science.

Taphonomic Factors	**Soil Science Metrics**
Physical	
Temperature	Soil Temperature Regime
Water movement	Soil Moisture Regime
	Slope
	Texture
Animal Factors	N/A
Burial depth	Soil Horizon
Chemical	
Mineral content	Cation Exchange Capacity
	Ion Content Values
Soil pH	Soil pH
Disolved gasses	Soil Horizon
Microbial	
Microbial activity	Organic Carbon Content
Organic matter	Organic Matter Content

Figure 3.2: Temperature regimes in the United States. Temperature regimes of the United States depicted on the U.S. map. Image provided by USDA/NRCS. http://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/use/maps/?cid=nrcs142p2_053998



There are twelve major soil taxonomic classes [8, 30]. These are categorized based on variables such as geographic region (temperature and moisture parameters), mineral and ion content, texture, and organic matter content [30]. These soil orders have been mapped to allow visual assessment of their prevalence per geographic region (Figure 3.3). All twelve soil orders can be found in the United States; however, some such as Ultisols are more prevalent in the Southeast region of the U. S. than others. With respect to forensic taphonomy, each of these soil orders can be defined based on mineral content, cation exchange capacity (CEC), texture, and organic matter content. CEC describes the availability of negatively charged ions in the soil for interaction with the cadaver. The higher the cation exchange capacity, the more cations the soil can hold. Soil cations include calcium, magnesium, potassium, ammonium, hydrogen, and sodium. Soil anions include chlorine, nitrate, sulfate, and phosphate. These cations and anions make up the mineral content of a soil, which is dictated by the parent material. The soil texture provides information regarding the type and thus the soil's ability to retain moisture or the ability of water to move through soil. In the instance of a horticulture proxy soil for an organic soil, this value is not available since it lacks minerals. Organic matter content affects the presence of microorganisms in the soil and soils can be classified based on their measured percentage of organic matter. Soil horizons with less than 20% organic carbon content are commonly considered mineral soils. Most of soil orders are mineral soils with only a few exceptions [30].

Figure 3.3: Soil classifications in the United States depicted on the United States continental map. Soil classification in the United States depicted on the U.S. map. Image provided by USDA/NRCS. Ultisols are represented by the yellow region and are common to the Southeastern United States. Access to Histosols was limited; therefore, a horticulture mix was used instead.

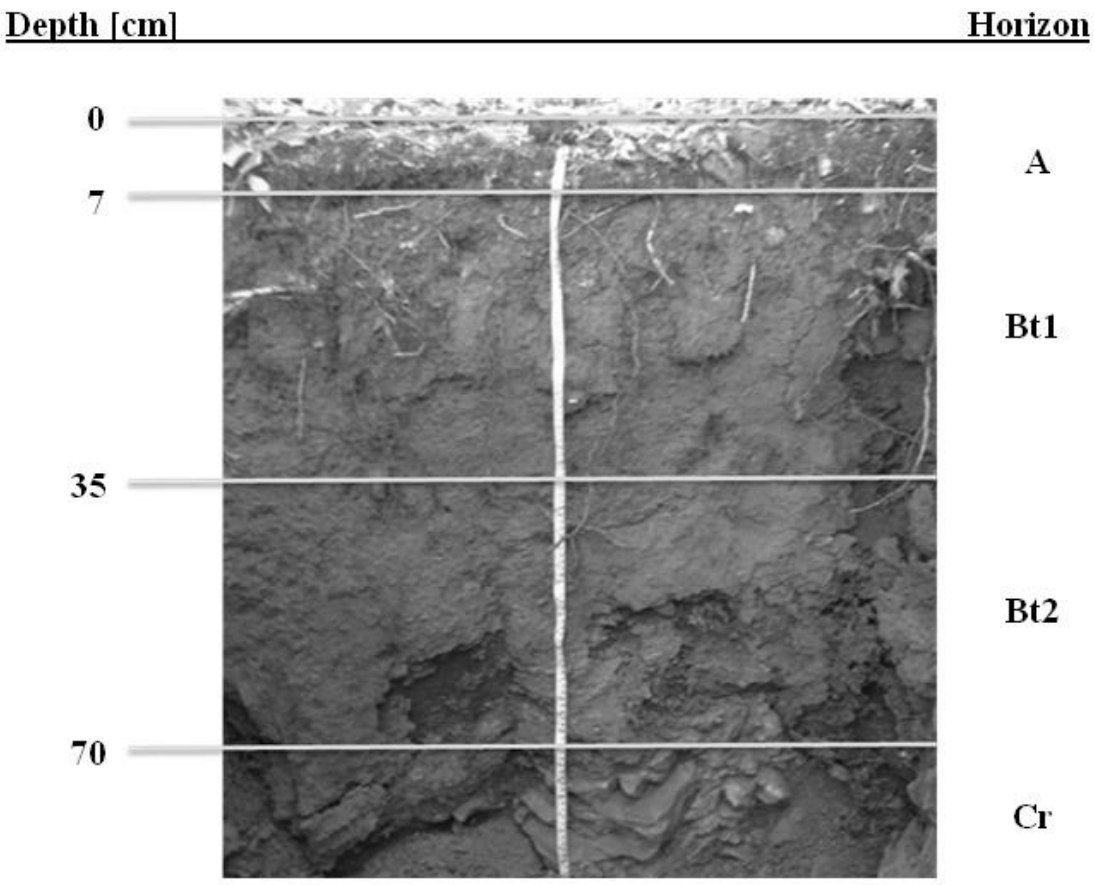
Soils found at different depths are defined as soil horizons and are characterized based on additions to and alterations of the parent material of that soil. The deepest soil horizon is bedrock, which is also the parent material of soil. In soil science, these soils depths are defined as soil horizons and are characterized based on additions to and alterations of the parent material of that soil. There are master horizons: organic materials (O), zone of humus accumulation and eluviation (A), zone of maximum eluviation (E), zones of maximum illuviation (B), parent material (C), rock (R) [30]. Lower-cased letters are often used to indicate more specific properties. For example the following horizons and subcategories were identified at the Simpson Agricultural Station site in Pendleton, SC shown in Figure 4. The transitional layer for the accumulation of silicate clay (t) in the B horizon would be described as the Bt horizon respectively as shown in Figure 3.5.

Preparation of Bioreactor

Bioreactor chambers were designed using 8.5 x 12 x 6 cm3 polystyrene boxes. Each chamber was thoroughly cleaned, dried and then sterilized using an ethylene oxide autoclave at 120°C for 30 minutes. Based on the discretization results obtained for objective two, the experimental design included the following variable conditions: four temperature regimes (Frigid, Mesic, Thermic, Hyperthermic); three soil types including two soil horizons of mineral soil and a sterilized organic soil (mineral – Ultisol, A horizon, mineral – Ultisol, Bt2 horizon, organic - horticulture mix, organic - horticulture mix sterilized); and two bone types (whole rib, thin section of rib).

Figure 3.4: Ultisol soil collection site at Simpson Agricultural Station, Pendleton, SC, USA.

Figure 3.5: Soil depths (cm) and horizons for collection of Ultisol at Simpson Agricultural Station. Soil depths (cm) and horizons for collection of Ultisol at Simpson Agricultural Station (A=zone of humus accumulation and eluviation; Bt1 and Bt2=zones of maximum illuviation of clay (kaolinite); Cr=saprolite, which is a chemically weathered rock). Soil Map Unit: HaD, Hiwassee sandy loam, 10 to 15% slope (Taxonomic class: Fine, kaolinitic, thermic Rhodic Kanhapludults).



Mineral soil was obtained at the Simpson Agricultural Station in Pendleton, SC site shown in Figure 3.4. This soil was from a Hiwassee sandy loam having a 10 to 15% slope and the taxonomic class: fine, kaolinitic, thermic Rhodic Kanhapludults. A kaolinitic soil consists of layered silicate mineral clay. A thermic soil temperature regime is in the range of 15 – 20 °C.

Due to the relative unavailability of organic soils in the southern United States, a commercially available horticulture mix (Vigoro® all-purpose potting mix) was used as a proxy for an organic soil. Horticulture mix soils generally incorporate high organic matter soils to promote plant growth. A sample of organic, horticulture mix was sterilized using ethylene oxide sterilization at 120°C for 30 minutes to kill any present microbial activity and used to determine if this sterilization would kill existing microorganisms in soil. Results of sample analysis are shown in Table 3.2.

A total of 14 chambers were created to simulate environmental factor combinations and are shown in Table 3.3. Eight of these chambers were used for thin section of rib bone tested in both mineral and organic soils for all four temperature conditions. For the remaining six chambers, the Frigid and Hyperthermic temperature conditions were utilized to simulate the effects of temperature extremes. Four of these held whole rib bones buried in B horizon mineral soil and organic soil, and two of these chambers simulated surface burial of thin sections of rib bone buried in A horizon mineral soil. All bioreactors were run for 60 days, and bone samples were collected at 30 day intervals.

Table 3.2: Analysis of bioreactor soils. Measurements are given for soils before use in the bioreactor experiment, as well as for 60 days after the experiment. For Ultisol calculations, n = 3 for each horizon. For horticulture mix calculations, n = 2.

	Soil Analysis - Averages	Standard Deviation
Ultisol - Minearl Soil - Horizon A		
Texture	Coarse sandy loam (65% sand+20% slit+15% clay)	N/A
Soil pH (pH) [-]	4.0	0.1
Cation Exchange Capacity (CEC) [meq/100g]	8.1	0.6
Organic Matter (OM) [%]	14.1	0.7
Calcium Ion Content [mg/kg]	108.0	27.1
Ultisol - Minearl Soil - Horizon B		
Texture	Clay (24% sand+18% silt+58% clay)	N/A
Soil pH (pH) [-]	5.4	0.1
Cation Exchange Capacity (CEC) [meq/100g]	4.9	0.3
Organic Matter (OM) [%]	12.2	1.2
Calcium Ion Content [mg/kg]	38.0	5.5
Horticulture mix - Organic Soil		
Texture	N/A	N/A
Soil pH (pH) [-]	5.8	0.1
Cation Exchange Capacity (CEC) [meq/100g]	16.4	0.8
Organic Matter (OM) [%]	74.5	3.0
Calcium Ion Content [mg/kg]	1373.0	87.0

Temperature chambers for the bioreactors were simulated using the following procedures. Frigid and Mesic conditions were achieved using separate laboratory refrigerators with variable temperature controls (Isotemp, Thermo Fisher Scientific Inc.) Thermic conditions were achieved by placing an insulated heating system set to 35°C inside of an environmental chamber set to 5°C. Finally, a hyperthermic environment was achieved using a laboratory incubator (Isotherm, Thermo Fisher Scientific Inc.) set to 38°C for the duration of the experiment.

Selection of Specimens for Use in Forensic Bioreactor

The impact of soil properties on forensic bone was simulated through burial of uniform bone samples in the bioreactors' soil. Fresh-frozen porcine spare rib bones were obtained from a local grocer in Greenville, SC and soft tissues were promptly removed and discarded. Porcine rib bone cross-sections (2 - 4 mm) were cut using a water-cooled band saw, rinsed in filtered water to remove residual blood, and air dried. One 8 cm porcine rib section was placed into five bioreactor environments and twelve thin sections were placed into a total of ten bioreactor environments. All bones were covered such that all surfaces were exposed to soil conditions. At predetermined time intervals of 30 and 60 days after burial, one thin section of bone was removed from each bioreactor making it available for subsequent assessment of bone morphology or biochemistry.

Table 3.3: Bioreactor experimental design. Experimental set up of the bioreactors' conditions was based on temperature regime (abbreviated as F, M, T, H for frigid, mesic, thermic and hyperthermic, respectively), soil horizon (abbreviated as A for surface grave A horizon and, B for shallow grave depth in Bt2 horizon), specimen type (abbreviated as W for whole or S for sections), and soil order (abbreviated as Min for mineral or Org for organic). The naming scheme and contents for the main categories of forensic bioreactor conditions are: S-Min-B) rib bone sections in mineral soil at shallow grave depth; W-Min-B) whole rib bone in mineral soil at shallow grave depth; S-Min-A) rib bone sections in mineral soil at surface grave depth; S-Org) rib bone sections in organic soil; and W-Org) whole rib bone in organic soil.

	Forensic Bioreactor Conditions				
Temperature Regime	S-Min-B	W-Min-B	S-Min-A	S-Org	W-Org
Frigid (0 - 7°C)	S-F-Min-B	W-F-Min-B	S-F-Min-A	S-F-Org	W-F-Org
Mesic (8 - 14°C)	S-M-Min-B			S-M-Org	
Thermic (15 - 21°C)	S-T-Min-B			S-T-Org	
Hyperthermic (>22°C)	S-H-Min-B	W-H-Min-B	S-H-Min-A	S-H-Org	W-H-Org

Monitoring of Bioreactor Conditions

Soil temperature was allowed to equilibrate for one week prior to placement of bone samples. Once at equilibrium, air temperature was measured using a digital thermometer. Frigid and Hyperthermic condition simulators were monitored by continuous digital readout recorded weekly. Mesic and Thermic conditions simulators were measured using a digital thermometer (Accumet Basic A815, Fisher Scientific) also measured weekly. The temperature conditions were monitored for the duration of the experiment (60 days).

Soil conditions were evaluated prior to addition of soil solution to bioreactor chambers at the Clemson Soil Analysis facility. Soil analysis of mineral soils at horizons A and Bt2, organic soil and autoclaved organic soil were conducted in triplicate, including basic evaluation as well as determination of organic matter content. These results are shown in Table 3.2

Results

Specification of Forensic Environmental Factors With Respect to Soil Science Metrics

Based on the systematic review of taphonomic, soil science and forensic literature, a series of variables were determined and aligned with appropriate soil science metrics presented in Table 3.1. Physical metrics were most directly related to soil taxonomy and mapped soil variable regimes. Soil taxonomy identifies soil orders and horizons, which then allow for analysis of burial depth and components of water movement such as slope and soil texture. Mapped soil variable regimes, for example the

soil moisture regime data provided averaged global moisture data based on location; whereas, soil slope and texture are obtained through site analysis and subsequent soil analysis, thus providing temporal site specific data. Similar maps are available for temperature. Based on this information, all four physical taphonomic factors were able to be applied to bioreactor design and were selected and controlled throughout a study.

Chemical metrics were most directly related to data obtained through soil science analysis. Soil analysis data can be obtained through both field and laboratory work. Soil horizon information related to depth of burial, which also corresponds to aerobic or anaerobic decomposition. The remaining chemical data metrics can be obtained through laboratory soil science analysis. For example, this analysis provides information regarding soil pH, one of the known taphonomic factors. For assessment of mineral content, there were two described soil science metrics obtained through soil analysis: cation exchange capacity (CEC) and soil ion content values. CEC describes the maximum quantity of total cations (e.g. Ca^{2+}) the soil is capable of holding. Based on this review, mineral content and soil pH can be easily determined before, during, or after bioreactor design and use. Soil taxonomy can be utilized to anticipate these variables prior to design of the research study. Soil analysis can be destructive or non-destructive depending on the research design. In contrast, presence of dissolved gasses can be easily anticipated in natural conditions; however, disturbance of the soil will aerate it upon placement in a bioreactor system and thus this variable is not a suitable bioreactor control.

Microbial metrics were related to organic matter and organic carbon values. Organic matter is directly measurable through soil laboratory analysis to obtain numerical values. This metric can also be determined using soil order and horizon data. Of the determined factors by forensic taphonomy, microbial activity is most affected by other outlined factors, and thus direct measurement would be an effective dependent variable for a study; however, as a result it cannot be considered a controllable environmental factor for the purposes of this study. In contrast, soil organic matter can be determined using soil taxonomy prior to initiation or research, and can be measured throughout the study through the use of soil laboratory analysis.

Discretization of Soil Science Metrics

Soil temperature was discretized using documented soil temperature regimes present in the United States shown in Figure 3.2. The main temperature regimes used in this study are Frigid, Mesic, Thermic, and Hyperthermic. The average temperature range for reach of these conditions is shown in Table 3.4. These temperature ranges were utilized to discretize temperature conditions for bioreactor design through the selection of a target temperature in each range.

Soil type was discretized through the use of soil taxonomy based on organic matter content. Soils above 20% organic carbon content are considered organic, and of the twelve soil orders, Histosols are the primary organic soil order. Thus, soil organic matter can be discretized into two primary categories, organic and mineral soils. Using

the map provided in Figure 3.3, soil type can also be discretized based on region and soil order to allow control of soil properties.

Burial depth can be discretized based on soil horizon. Soil horizons are specific to locations and define the state of altered parent material as it moves from rock to soil form. An example of soil horizons measured at the Simpson Agricultural Facility (Figure 3.4) is shown in Figure 3.5. In this Ultisol, the soil has been discretized into four specific horizons: A, Bt1, Bt2, and Cr. For the purpose of forensic research, a surface scatter could be defined as located in the A horizon, thus specifying the exposed soil conditions. Similarly, a shallow grave burial at 50 cm could be defined as a part of the Bt2 horizon.

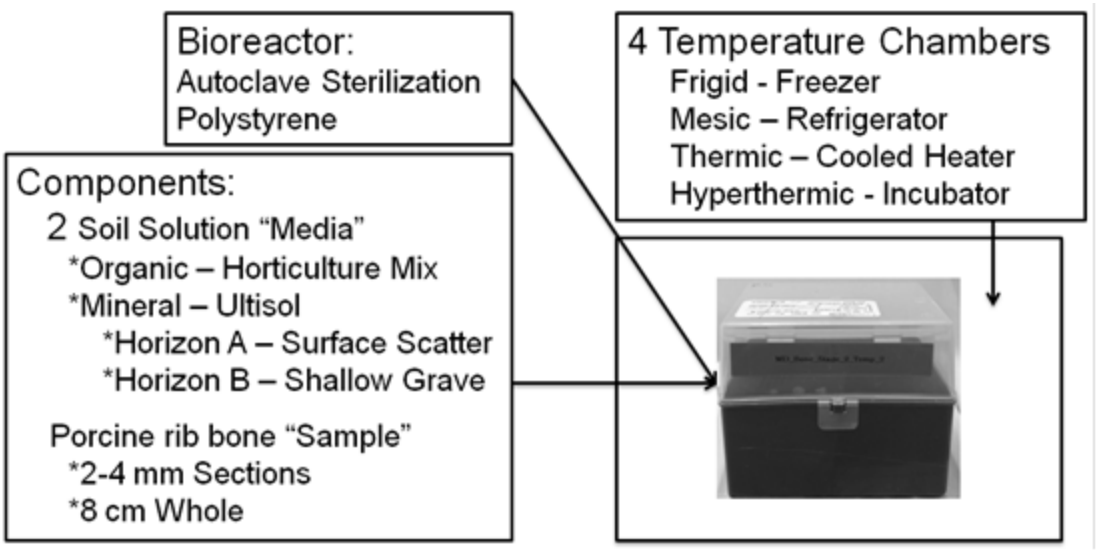
Validation of Forensic Bioreactor Inputs

The final bioreactor design incorporates a temperature chamber based on the four predetermined temperature regimes (Figure 3.6). The simulated temperature chambers conditions were Frigid (3.8 ± 0.8°C), Mesic (8.8 ± 0.9°C), Thermic (16.0 ± 1.0°C), and Hyperthermic (37.5 ± 0.1°C) (Table 3.4). The internal components of the bioreactor were the soil solution and the porcine bone specimens simulating skeletal remains in gravesoil. Analysis of soil solutions confirmed that discrete characteristics of mineral and organic soils at different burial depths were analyzed and reported in Table 3.2 This table reports the texture, soil pH, CEC, organic matter content, and calcium ion content for each soil type used in this study.

Table 3.4: Bioreactor temperature environments based on established temperature regimes. Temperature regimes of the United States compared to bioreactor environment temperatures created in the laboratory and monitored throughout the 60 day period of this study.

Soil Temperature Regime	Regime Range [°C]	Simulated Average [°C]	Standard Deviation [°C]
Frigid	0 -- 7	3.8	0.8
Mesic	8 -- 14	8.8	0.9
Thermic	15 -- 21	16.0	1.0
Hyperthermic	>22	37.5	0.1

Figure 3.6: Bioreactor system discretization and design. Final bioreactor system containing a temperature chamber, bioreactor chamber, and environmental components (soil type, forensic specimen).



Results of soil analysis show the soil pH to be between 4 – 6 for all soils analyzed. In contrast, The CEC of Ultisol at horizon Bt2 was lowest at 4.9 meq/100 g. CEC for Ultisol at horizon A was 8.1, and the CEC of the horticulture mix was 16.4. Differences in calcium ion content and organic matter content were most stark between mineral and organic soils. The Organic matter contents of the two Ultisol horizons were 14.1% and 12.1%, while the organic matter content of the organic soil was 74.5%. Similarly, the calcium ion content of the Ultisol horizon A and B were 108.0 and 38.0 mg/kg respectively, while the calcium ion content of the organic soil was 1373.0 mg/kg. This dramatic increase may be due to soil enrichment for horticulture purposes; however, such dramatic increase may provide insight into the effect of calcium content on decomposition.

Discussion

The study describes an interdisciplinary approach to forensic anthropology and develops a forensic bioreactor system based on defined factors known to impact bone decomposition (Figure 3.1). Specifically, environmental factors important to forensic anthropology and useful for modeling decomposition in an outdoor context were defined and then discretized using soil science data. Finally, these factors were successfully incorporated into an easily monitored forensic bioreactor prototype that mimicked an outdoor setting. In this manner, the bone samples were treated as a tissue undergoing dynamic changes when exposed to a soil medium. This research provides a foundation

for bone decomposition models and advances forensic anthropology related to determining PMI for skeletal remains.

The environmental variables most commonly related to decomposition were identified and related to the corresponding soil science metrics. Of these variables, moisture, burial depth, mineral content, soil pH, and organic matter content were identified as pre-experiment parameters. These variables can be identified through existing soil science literature and many of which are easily assessable through the use of online databases, allowing for the appropriate selection of soil to fit the researchers need. In addition, these variables can be quantified before, during or after the experiment depending on the objectives of the research. Temperature and evaluation of dissolved gas content were identified as variables that could be measured and controlled during the experiment using common laboratory equipment. Using existing databases, these variables were discretized to allow testing of specific environmental conditions across a global spectrum of potential environments (Figure 2 and Figure 3). In this manner, it is possible to develop a bioreactor system capable of simulating a broad range of temperature and soil related environmental conditions.

Other variables identified here that were not simulated in this study can be discretized using similar means. Soil moisture can be discretized based on the soil moisture regimes also provided by the USDA, and monitored using soil analysis facilities. Mineral content and soil pH can also be selected for based on soil order using Figure 3.3 or through analysis of local soils. Each soil order has specific attributes such as predominant minerals that are commonly denoted as sub-classifications.

The bioreactor systems designed in this experiment were able to simulate four different temperature conditions within 1°C of the target temperature, two extremes of soil organic matter content, and two types of forensic burial scenarios. In addition, test samples were selected that accounted for two levels of exposure that could be anticipated in a clandestine burial of human remains. Bioreactor chambers were easily sterilized prior to research, as well as constructed of disposable materials to facilitate post-experiment disposal. Through the use of clear, latching lids, each chamber was also easily accessible for collection of samples over the course of the experiment. This study was limited in the number of variables that could be incorporated into the system. Modifications to this system would allow for inclusion of additional variables, as well as make the system easier to design in non-university laboratories.

Future bioreactor systems may incorporate a complete seal to allow the creation of a constant atmospheric environment. This type of design would allow for the control of exposure to gasses such as oxygen and carbon dioxide, which can directly determine if decomposition will be driven by anaerobic or aerobic organisms. In addition, a sealed system would allow for the implementation of a desired moisture scheme. A moisture scheme could involve addition of water at a constant rate over the experiment to test water movement, or the continuous measurement of moisture levels with addition of water if levels drop below a desired level. It would also be important to utilize glass rather than polystyrene chambers. Glass can be sterilized using simpler method than plastics, which would make this type of system easier to design.

Ultimately, this research has shown the potential for collaborations between forensic research, soil science, and engineering to develop a system that allows the simulation of many environments in one research location. Through the alignment of existing variables with those measurable in other fields, forensic research can focus on quantitative evaluation of research questions. This system is particularly valuable for the testing of new research methods in forensic taphonomy that are limited by the availability of funding to test their ideas globally.

CHAPTER FOUR

ANALYSIS OF PORCINE RIB BONES AGED IN FORENSIC BIOREACTORS

Introduction

Through application of the methodologies developed in Chapters 2 and 3, this chapter will aim to test several questions currently surrounding the citrate method. These questions include what causes citrate to biochemically degrade in postmortem bone exposed to a gravesoil environment and can the citrate method in bone be applied to bones found in environments other than those found at the University of Tennessee [26]. Based on the results of Kanz et al. (2013), the mathematical model used to calculate PMI using citrate concentration cannot be successfully applied to samples found in a different environmental context [17]. Further testing is needed in order to evaluate the potential of the citrate method. This study will utilized forensic bioreactors to conduct a small scale test considering multiple environment factors at one laboratory site to determine the parameters for a large scale test.

This study considered four primary experimental parameters: temperature, soil type, and sample exposure. Each of parameter was tested using the bioreactors designed in Chapter 3. The discretized variables considered include: four temperature regimes (Frigid, Mesic, Thermic, and Hyperthermic), two soil organic matter content levels (mineral and organic), and three types of exposure (solution, thin sectioned porcine rib

bone, and whole porcine rib bone). The study was conducted over a 60 day time period. Collected samples were analyzed using the chemical methods outlined in Chapter 2.

Based on the research conducted in Chapter 2, several hypotheses were developed based on the three parameters to be tested. With respect to the environmental parameter temperature, it is hypothesized that exposure to higher temperatures over sixty days will lead to higher loss of citrate in porcine rib bone. With respect to the environmental parameter soil organic matter content, it is hypothesized that exposure to higher soil organic matter content will result in higher microbial activity, and thus lead to higher loss of citrate in porcine rib bone over sixty days. With respect to the environmental parameter sample exposure, it is hypothesized that higher levels of exposure will lead to higher loss of citrate in porcine bone over sixty days.

Materials and Methods

Application of Bioreactor System

The hypothesis regarding the effect of temperature was tested through the exposure of citrate solution and citrate thin section mineral soil bioreactors to four temperature conditions. These bioreactors are indicated by the X-Sol abbreviations and S-X-Min-B abbreviations in Table 4.1, where X represents a given temperature condition. They are shown in columns 1 and 2 for all rows.

The hypothesis regarding the effect of soil organic matter content was tested through the exposure of thin section porcine rib bone to mineral or organic soil

bioreactors. These bioreactors are indicated by the S-H-Min-B and S-H-Org abbreviations in Table 4.1. They are shown in columns 2 and 5 in row four.

The hypothesis regarding the effect of sample exposure was tested through the use of three different sample types: citrate in solution, thin sections of porcine bone, and whole porcine bone. Citrate was suspended in Nanopure water to create a citrate solution. Thin sections and whole bone were suspended in mineral soil from horizon Bt2. These bioreactors are indicated by the H-Sol, S-H-Min-B, and W-H-Min-B abbreviations in Table 4.1. They are shown in columns 1, 2, and 3 in row four.

Application of Chemical Testing Methods

Samples were collected from bioreactors at 0, 30, and 60 day time points. All samples were stored at -10°C until preparation using the sample preparation protocol outlined in Chapter 2. During this preparation, image data was taken upon collection from the bioreactor and after reducing the bone to a fine powder. After sample preparation from sectioned bone to a solution, the collagen pellet and supernatant were stored separately. The supernatant was then tested using the Abcam citrate assay based on the calibration methods used in Chapter 2. After data was collected utilizing colorimetric assay techniques, HPLC measurement was conducted as shown in Chapter 2 using an external laboratory.

Table 4.1: Small scale bioreactor experimental set-up. Experimental set up of the bioreactors' conditions was based on temperature regime (abbreviated as F, M, T, H for frigid, mesic, thermic and hyperthermic, respectively), soil horizon (abbreviated as A for surface grave A horizon and, B for shallow grave depth in Bt2 horizon), specimen type (abbreviated as W for whole or S for sections), and soil order (abbreviated as Min for mineral or Org for organic). The naming scheme and contents for the main categories of forensic bioreactor conditions are: S-Min-B) rib bone sections in mineral soil at shallow grave depth; W-Min-B) whole rib bone in mineral soil at shallow grave depth; and S-Org) rib bone sections in organic soil.

		Forensic Bioreactor Conditions			
Temperature Regime	Sol	S-Min-B	W-Min-B	S-Min-A	S-Org
Frigid (0 - 7°C)	F-Sol	S-F-Min-B			
Mesic (8 - 14°C)	M-Sol	S-M-Min-B			
Thermic (15 - 21°C)	T-Sol	S-T-Min-B			
Hyperthermic (>22°C)	H-Sol	S-H-Min-B	W-H-Min-B	S-H-Min-A	S-H-Org

Results

Images of bone powder

Images were collected of bone after crystals after processing with liquid Nitrogen. Images were collected using a light microscope at a magnification of 50x using an Infinity 2 digital camera (Lumenera Corporation, ON, Canada). Images are shown in Table 4.2. Target crystal size was <10 μm. Bone fragments in these images represent powder sizes across eight pone powder preparations. Qualitative assessment shows bone fragments below10 μm. Several images show fragments above the target fragment size. In these instances, bone samples were reprocessed with liquid nitrogen.

Chemical Assay Results

Initial porcine rib bone citrate solution samples were initially prepared using both cortical and trabecular bone. Test category 1 in Figure 4.1 represents citrate concentration measurements of samples prepared this way. The average sample solution citrate concentration was 1.1 wt. %. Test category 2 in Figure 4.1 represents citrate concentration measurements of samples prepared using only cortical bone during sample preparation. The average sample solution concentration of these samples was 1.7 wt. %. Test category 3 represents the measurement of porcine rib bone citrate concentrations from the cortical bone of six porcine rib bones tested in duplicate. These citrate concentrations were significantly lower than those of previous sample measurements. The average sample solution concentration was 0.2 wt. %.

Table 4.2: Imaging of powdered bone samples.

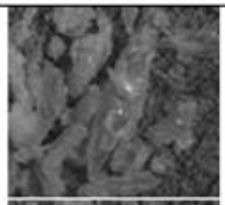
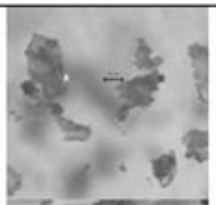
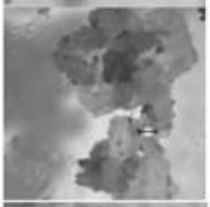
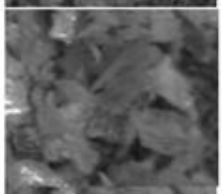
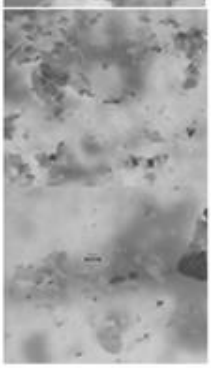
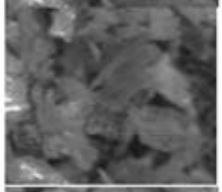
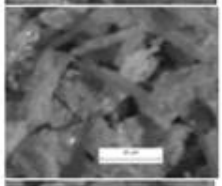
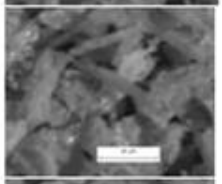
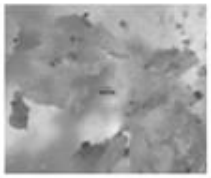
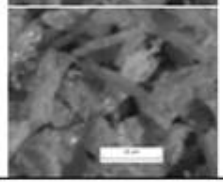
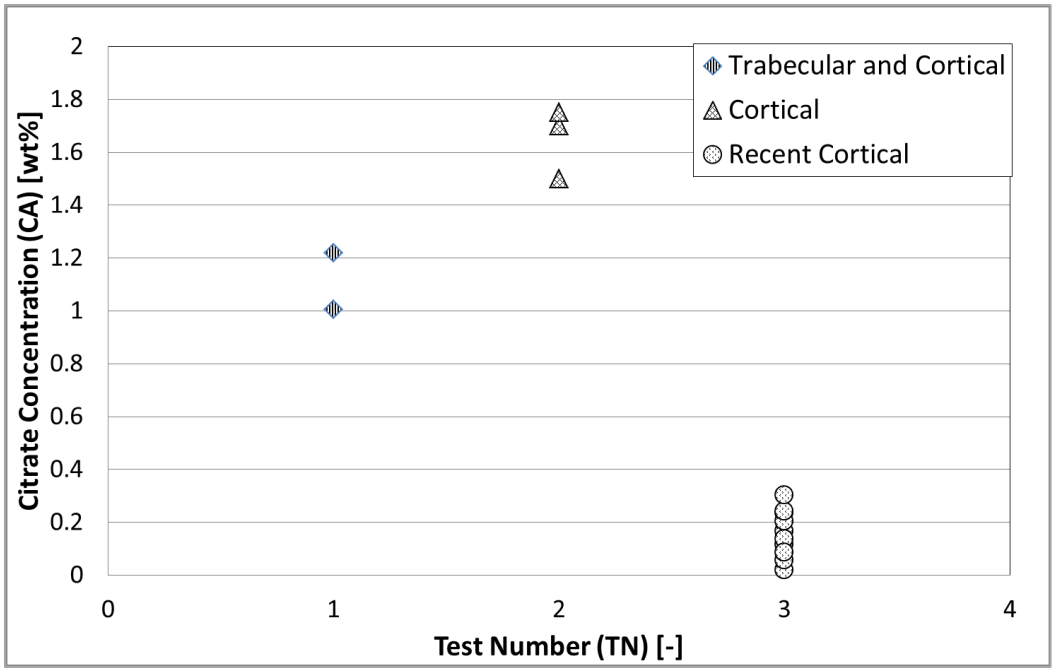
Bioreactor Condition	0 Days	30 Days
S-F-Min-B		
S-M-Min-B		
S-T-Min-B		
S-H-Min-B		
S-H-Org		
S-H-Min-A		
W-H-Min-B		

Figure 4.1: Citrate concentrations of fresh porcine rib bone. Samples represented here were conducted in three separate preparations of fresh porcine rib bones. Samples shown in test number 1 were prepared using whole porcine rib bone sections (trabecular and cortical bone). Samples shown in test number 2 represent samples were cortical bone was removed from trabecular prior to powdering. Samples shown in test number 3 represent samples prepared from six fresh porcine rib bones that demonstrate measurement complications and rejected results due to a citrate concentration below the minimum threshold for consideration in this research for fresh porcine bone (1 wt. %).



Sample solutions utilized to test hypothesis one, the effect of temperature, were assayed to determine citrate concentrations. Citrate solution samples were collected from each of four temperature conditions and citrate concentration was measured using the Abcam citrate assay (Cambridge, MA). These results are shown in Figure 4.2. A single point is shown for the zero time point as a single stock solution was utilized in all temperature conditions. Similarly, fresh bone samples were collected from each of four temperature conditions, processed using the protocol shown in Figure 2.2, and citrate concentration was measured using the Abcam citrate assay (Cambridge, MA). These results are shown in Figure 4.3. It can be seen that thirty day samples were measured to have lower citrate concentrations than sixty day samples. After measurement using the citrate assay, these samples prepared from porcine rib bone were also measured using HPLC. The results of HPLC measurement are shown in Figure 4.4

Bone sample utilized to test hypothesis two, the effect of soil type, were collected from the Hyperthermic temperature condition, and citrate concentration was measured using the Abcam citrate assay (Cambridge, MA). These results are shown in Figure 4.5. Sample data is currently unavailable for the organic soil – sixty day measurement point. In addition, sample data for the mineral soil – thirty day measurement point resulted in a zero value and is represented as such in Figure 4.5. After measurement using the citrate assay, these samples were measured using HPLC. The results of HPLC measurement are shown in Figure 4.6.

Figure 4.2: Change in citrate concentration over time of citrate solutions exposed to four temperature conditions. Samples represented here were prepared with granular sodium citrate and exposed to one of four temperature conditions (Frigid, Mesic, Thermic, Hyperthermic) over sixty days. Results show a decrease in citrate concentration with time, with an increasing rate of decrease as exposure temperature increases.

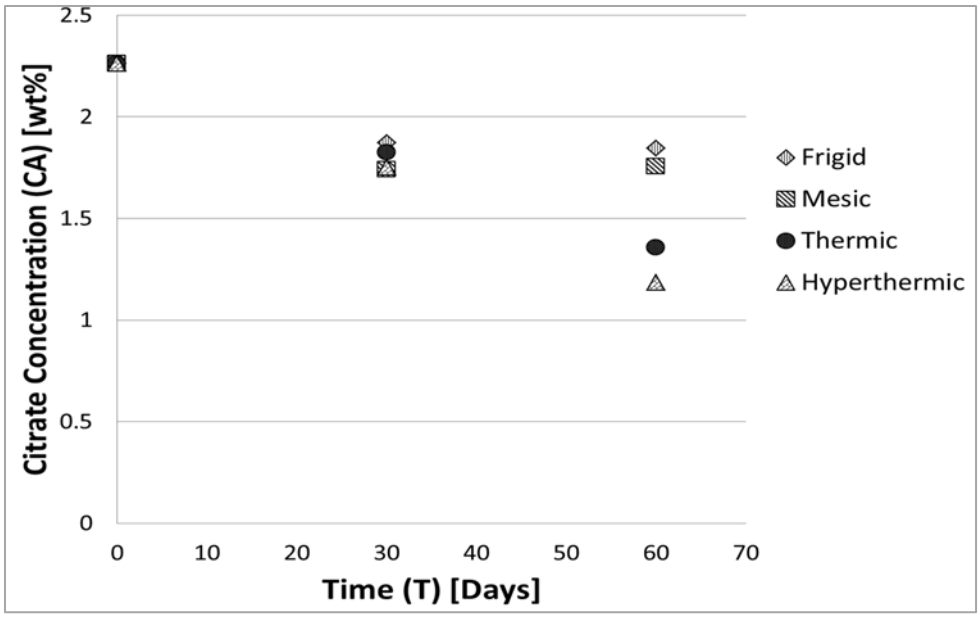


Figure 4.3: Change in citrate concentration over time of porcine rib bone samples exposed to four temperature conditions and measured by colorimetric assay. Samples represented here were porcine rib bone sections (2-4 mm) that were exposed to one of four temperature conditions (Frigid, Mesic, Thermic, Hyperthermic) over sixty days. Results show a decrease in citrate concentration from time 0 day measurements; however, results considering 30 and 60 day time measurements were inconclusive.

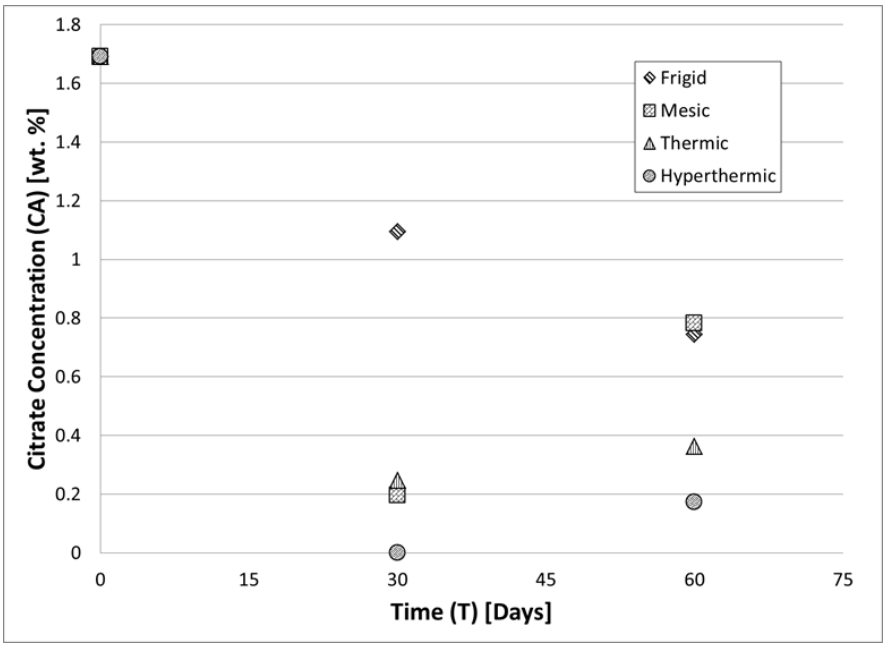


Figure 4.4: Change in citrate concentration over time of porcine rib bone samples exposed to four temperature conditions and measured by HPLC. Samples represented here were porcine rib bone sections (2-4 mm) that were exposed to one of four temperature conditions (Frigid, Mesic, Thermic, Hyperthermic) over sixty days. Results show a decrease in citrate concentration from time 0 day measurements; however, results considering 30 and 60 day time measurements were inconclusive.

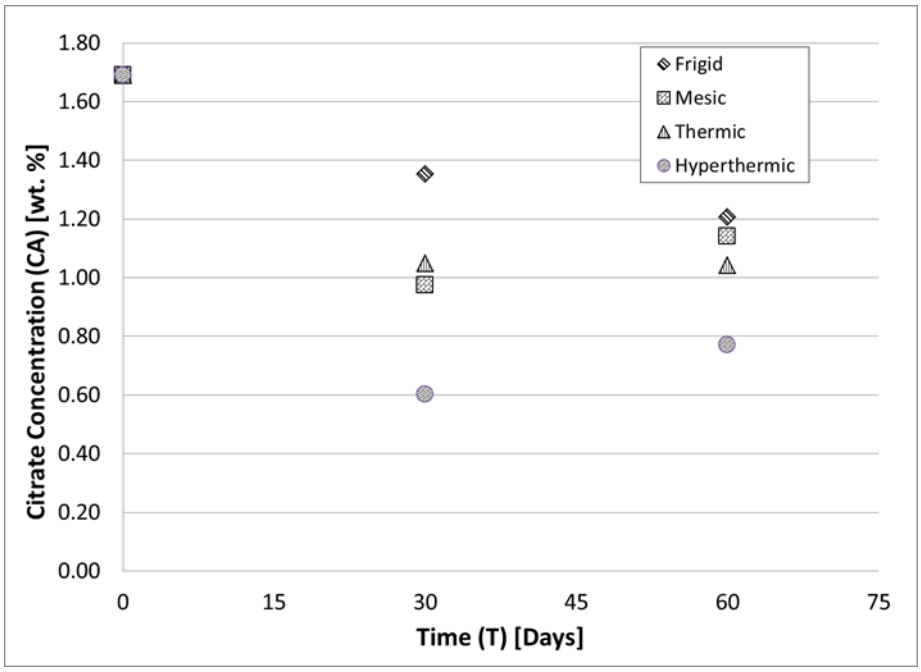


Figure 4.5: Change in citrate concentration over time of porcine rib bone samples exposed to two soil type conditions and measured by colorimetric assay. Samples represented here were porcine rib bone sections (2-4 mm) that were exposed to one of two soil organic matter content conditions (mineral (low organic matter) or organic (high organic matter)) over sixty days. Results show a decrease in citrate concentration from time 0 day measurements; however, results considering 30 and 60 day time measurements were inconclusive.

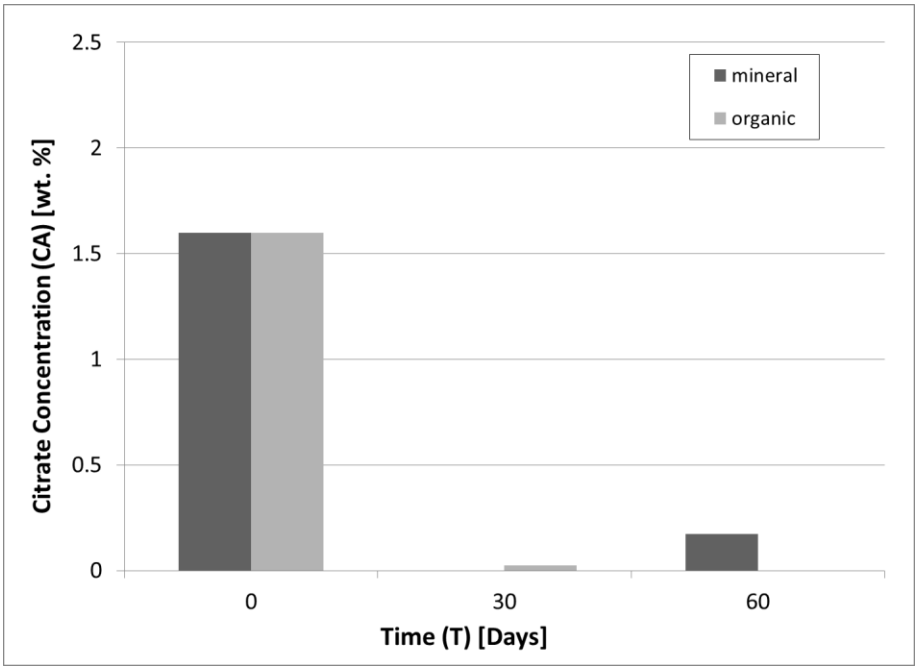
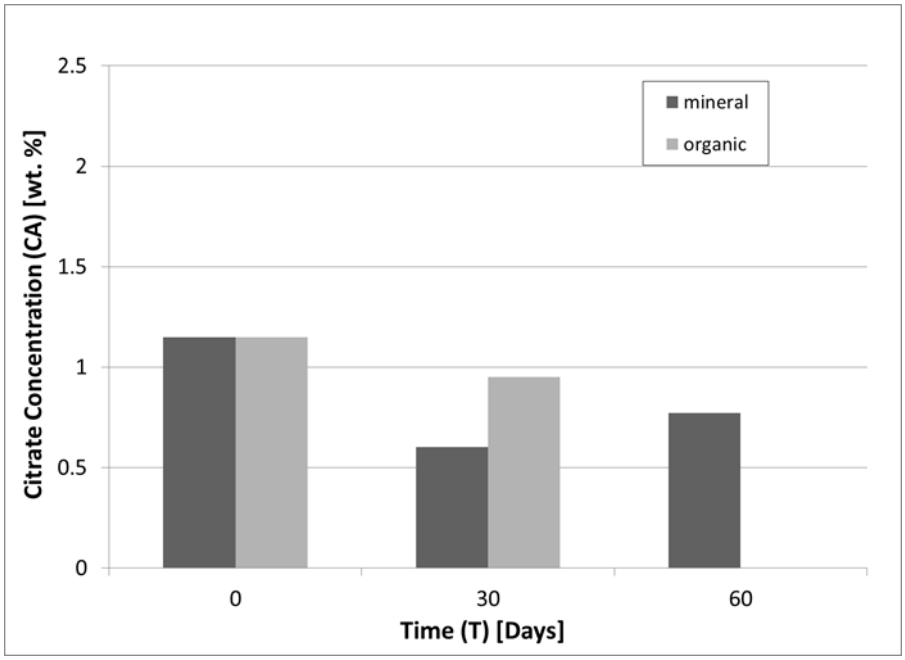


Figure 4.6: Change in citrate concentration over time of porcine rib bone samples exposed to two soil type conditions and measured by HPLC. Samples represented here were porcine rib bone sections (2-4 mm) that were exposed to one of two soil organic matter content conditions (mineral (low organic matter) or organic (high organic matter)) over sixty days. Results show a decrease in citrate concentration from time 0 day measurements; however, results considering 30 and 60 day time measurements were inconclusive.



Bone sample utilized to test hypothesis three, the effect of sample exposure, were collected from the Hyperthermic temperature condition, and citrate concentration was measured using the Abcam citrate assay (Cambridge, MA). These results are shown in Figure 4.7. Sample data is currently unavailable for the whole bone – sixty day measurement point. In addition, sample data for the mineral soil – thirty day measurement point resulted in a zero value and is represented as such in Figure 4.7. After measurement using the citrate assay, these samples were measured using HPLC. The results of HPLC measurement are shown in Figure 4.8.

Citrate concentration is stated in the literature to be fixed at the time of death. Schwarcz et al. (2010) concluded that citrate decreases with time when exposed to soil at a temperature above 0°C [26]. The data collected from the six bioreactor systems presented here demonstrates citrate values both decreasing and increasing with time. A decrease in citrate concentration is seen between 0 and 30 day data points and 0 and 60 day data points for all bioreactors. An increase is seen in several cases between 30 and 60 day data points. These results are consistent between data obtained using colorimetric assay and HPLC. Therefore, the results presented here represent inconclusive data.

Conclusion

This pilot study demonstrated the use of forensic bioreactors with porcine rib bone specimens. In addition, it demonstrated an increase in the rate of decline of citrate suspended in solution when subjected to higher temperature conditions. Although this study resulted in inconclusive results regarding the decline of citrate bound in bone, a

Figure 4.7: Change in citrate concentration over time of citrate in solution, in 2 – 4 mm bone sections (section), or 8 cm bone sections (whole) and measured by colorimetric assay. Samples represented here were porcine rib bone sections (2-4 mm) or whole porcine rib bones (8 cm) that were exposed to mineral soil conditions over sixty days. Results show a decrease in citrate concentration from time 0 day measurements; however, results considering 30 and 60 day time measurements were inconclusive.

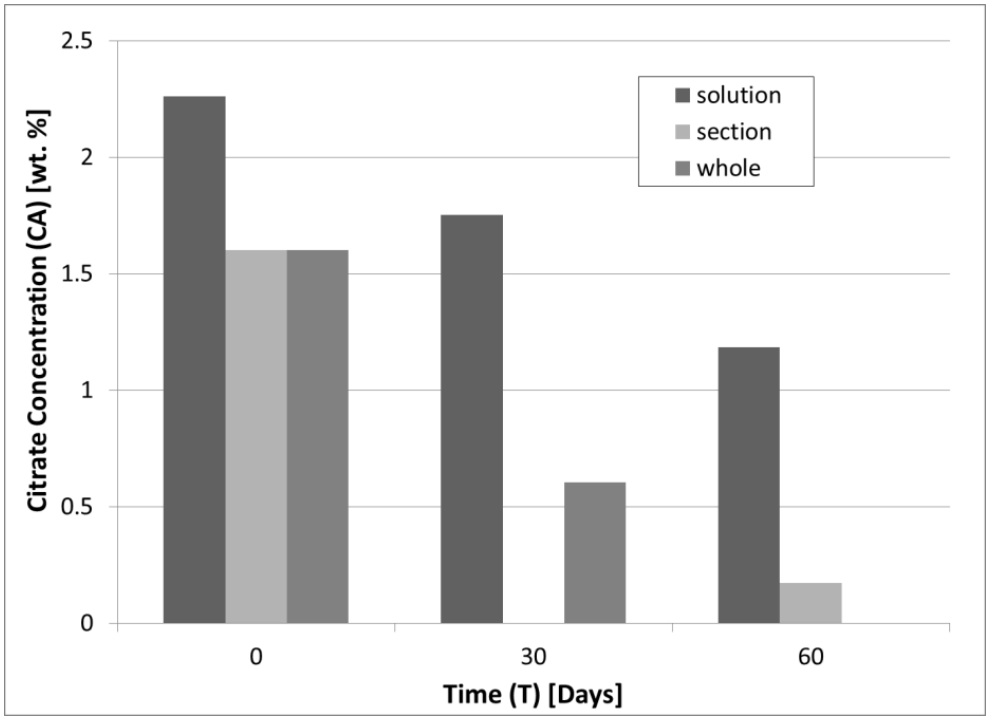
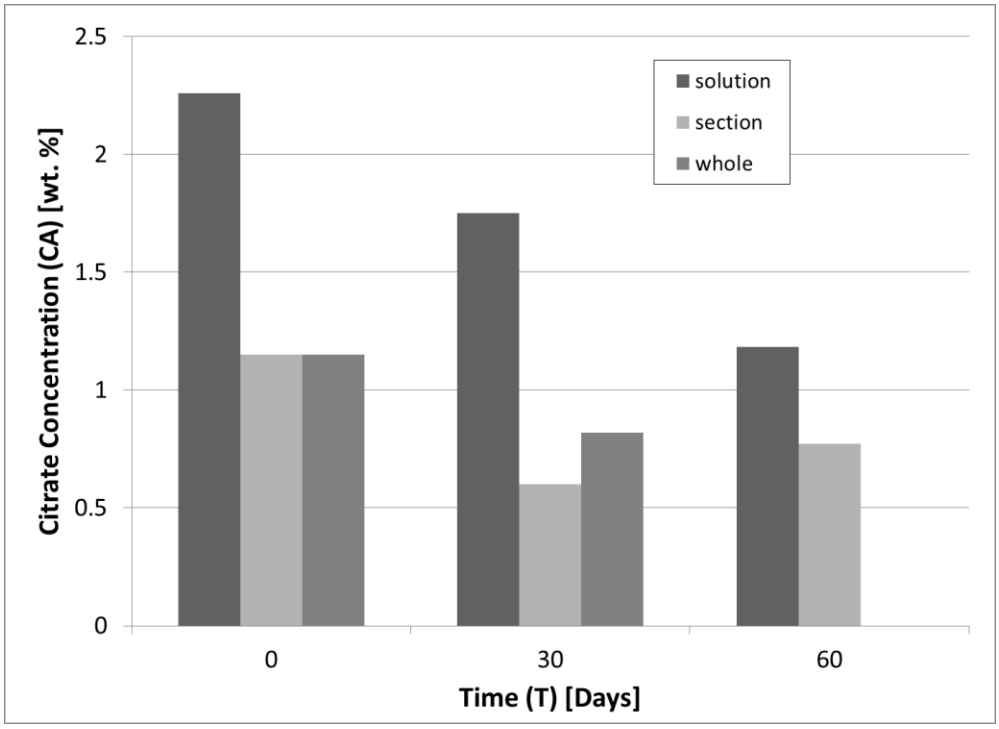


Figure 4.8: Change in citrate concentration over time of citrate in solution, in 2 – 4 mm bone sections (section), or 8 cm bone sections (whole) and measured by HPLC. Samples represented here were porcine rib bone sections (2-4 mm) or whole porcine rib bones (8 cm) that were exposed to mineral soil conditions over sixty days. Results show a decrease in citrate concentration from time 0 day measurements; however, results considering 30 and 60 day time measurements were inconclusive.



decline in citrate concentration was detected for all experiments compared to measurements taken before placement in the bioreactor system. Therefore, this study presents the need for continued refinement of the citrate analysis method, specifically with respect to the processing of bone samples.

Citrate analysis could not replicate the initial concentration values or a logarithmic citrate decline as demonstrated by Schwarcz et al. (2010) [26]. In addition, the results of Kanz et al. (2013) do not consider samples for PMI values between 0 and 60 days, and measured citrate concentrations are consistently found at less than 1 wt. % for samples of PMI greater than 30 years [17]. Based on the results presented here and the work by Kanz et. al (2010), it is possible that initial citrate concentrations of human and porcine bones are not consistently 2 wt. %.

In order to determine the initial concentration of citrate, additional research is needed to validate existing and potential methods for the calculation of postmortem bone citrate concentration. Existing methodology is not succinct enough to allow replication in practice by a forensic scientist. Once more robust methodology is in place, analysis of the environmental factors outlined here can be conducted to obtain conclusive results regarding the impact of the environment on the decline of citrate in bone. Through the evaluation of these environmental factors, the use of citrate to determine the PMI can move closer to application in practical forensic science.

CHAPTER FIVE

CONCLUSION

This research provides the basis for further development of the citrate method for the determination of the PMI. Through the further evaluation of existing tools for the measurement of citrate concentration and bone, as well as the development of a bioreactor system for the ageing of bone samples in a controlled environment, this research has set the ground work for the progression of the citrate method toward practical application. Additional studies are needed to apply these tools and to develop a model that can accurately calculate PMI from citrate concentration, irrespective of the environment in which the bone was discovered.

Chapter two of this study provided an extended methodology for the evaluation of new methods to measure citrate concentration in bone. Through the additional review of sample preparation, the proposed method of sample preparation provides increased opportunities for comparison of results. In addition, measurement of citrate concentration using colorimetric assay and HPLC techniques was tested in this chapter in order to identify challenges encounter when utilizing this methodology. This chapter systematically assessed the potential of the colorimetric assay, and compared these results to those obtained using HPLC. Although the expected concentration of citrate in bone was not found, this research presents then need for further validation of the results presented by Schwarcz et al. (2010) before this method can truly be applied in forensic science case studies.

Chapter three developed a new system for the preparation of samples to be used in forensic research. This chapter focused on identifying the variables previously identified to impact decomposition, and highlighted metrics that can be easily assessed during an experiment or selected for at the start of an experiment. A total of nine environmental factors were identified in this chapter as impacting decomposition, and eleven metrics were proposed for the assessment of these factors. Of these, six were implemented in a forensic bioreactor to simulate a clandestine burial. A total of fourteen systems were developed, each simulating a unique set of environmental parameters. These systems can be further modified to simulate any environment desired by the forensic scientist. Through the application of these systems, forensic research can conduct research studies on the in a laboratory setting to evaluate the need for higher resource, longer duration studies, thus reducing the initial research costs and allowing increased flexibility in experimental design.

Chapter four presented a pilot study using the bioreactors developed in Chapter 3 and assessing the samples obtained using the methodology developed in Chapter 2. Pilot study results were inconclusive; however, this study can confirm that citrate concentration declines after contact with a soil based environment. In addition, the bioreactor systems allowed easy access to samples and demonstrated environmental consistency throughout this study. Ultimately, this study showed the potential for the evaluation of multiple environmental conditions in a laboratory setting that would otherwise require travel to many different locations.

In conclusion, this study presents new methodologies for the continuation of forensic research. Two chemical techniques were assessed for application of the citrate method for the determination of the PMI, and it was concluded that additional research is needed to confirm the initial concentration of citrate in bone samples. Forensic bioreactor systems were developed that could simulate environments based on existing databases of environmental information and a method was developed for the application of this data. These forensic bioreactors were tested in a pilot study; however, due to the limitations of methodologies for the calculation of citrate concentration, these results were inconclusive. Future work is needed to verify the citrate measurement procedures outlined by Schwarcz et al. (2010) and revisited in this study. With this additional research, the forensic bioreactor system can be improved and applied to the citrate method to determine the impact of environmental variables that may be currently limiting the application of this method to studies such as that of Kanz et al. (2013). Ultimately, these new developments can provide new tools to forensic science through collaboration with soil science and bioengineering.

REFERENCES

1. Abdel-Maksoud, G. (2010). Comparison Between the Properties of ″ Accelerated-Aged ″ Bones and Archaeological Bones. Mediterranean Archaeology and Archaeometry, 10(1), 89–112.

2. Bell, L. S., Skinner, M. F., & Jones, S. J. (1996). The speed of post mortem change to the human skeleton and its taphonomic significance. Forensic Science International, 82, 129–140.

3. Carter, D. O., Yellowlees, D., & Tibbett, M. (2008). Temperature affects microbial decomposition of cadavers (Rattus rattus) in contrasting soils. Applied Soil Ecology, 40(1), 129–137. doi:10.1016/j.apsoil.2008.03.010

4. Carter, D. O., Yellowlees, D., & Tibbett, M. (2010). Moisture can be the dominant environmental parameter governing cadaver decomposition in soil. Forensic Science International, 200(1-3), 60–6. doi:10.1016/j.forsciint.2010.03.031

5. Child, A. M. (1995). Towards and Understanding of the Microbial Decomposition of Archaeological Bone in the Burial Environment. Journal of Archaeological Science, 22(2), 165–174. doi:10.1006/jasc.1995.0018

6. Collins, M. J., Nielsen-Marsh, C. M., Hiller, J., Smith, C. I., Roberts, J. P. (2002). The survival of organic matter in bone: a review. Archaeometry, 44(3), 383–394. doi:10.1111/1475-4754.t01-1-00071

7. Costello, L. C., Franklin, R. B., Reynolds, M. A., Chellaiah, M. (2012). The Important Role of Osteoblasts and Citrate Production in Bone Formation: "Osteoblast Citration" as a New Concept for an Old Relationship. The Open Bone Journal, 4, 27–34. doi:10.2174/1876525401204010027

8. Damann, F. E., Tanittaisong, A., & Carter, D. O. (2012). Potential carcass enrichment of the University of Tennessee Anthropology Research Facility : A baseline survey of edaphic features. Forensic Science International, 222, 4–10. doi:10.1016/j.forsciint.2012.04.028

9. Dent, B. B., Forbes, S. L., & Stuart, B. H. (2004). Review of Human Decomposition Processes in Soil. Environmental Geology, 45, 576–585.

10. Dickens, F. (1941). The Citric Acid Content of Animal Tissues , with Reference to Its Occurrence in Bone and Tumour. Biochemical Society of Sheffield, (1011).

11. Eeckhaut, A. Van, Lanckmans, K., Sarre, S., Smolders, I., & Michotte, Y. (2009). Validation of bioanalytical LC – MS / MS assays : Evaluation of matrix effects. Journal of Chromatography B, 877, 2198–2207. doi:10.1016/j.jchromb.2009.01.003

12. Figueiredo, M., Cunha, S., Martins, G., Freitas, J., Judas, F., & Figueiredo, H. (2011). Influence of hydrochloric acid concentration on the demineralization of cortical bone. Chemical Engineering Research and Design, 89(1), 116–124. doi:10.1016/j.cherd.2010.04.013

13. Gibbs, L. M. (1991). What' s Sex in the East is Not Necessary Sex in the West : Citrate , Sex and Human Skeletal Remains. McMaster University Open.

14. Guilbault, G. G., & Hjelm, M. (1989). IUPAC Gold Book. Pure and Applied Chemistry, 61(9), 1657–1664. doi:10.1351/pac198961091657

15. Hu, Y., Rawal, A., & Schmidt-Rohr, K. (2010). Strongly bound citrate stabilizes the apatite nanocrystals in bone. PNAS, 107(52), 22425–22429. doi:10.1073/pnas.1009219107

16. Jehle, S., Hulter, H. N., & Krapf, R. (2014). Effect of Potassium Citrate on Bone Density , Microarchitecture , and Fracture Risk in Healthy Older Adults without Osteoporosis : A Randomized. Endocrine Research, 98(January 2013), 207–217. doi:10.1210/jc.2012

17. Kanz, F., Reiter, C., & Risser, D. U. (2013). Citrate Content of Bone for Time Since Death Estimation : Results from Burials with Different Physical Characteristics and Known PMI *. Journal of Forensic Sciences, 1–8. doi:10.1111/1556-4029.12341

18. Knuuttila, M., Lappalainen, R., Alakuijala, P., & Lammi, S. (1985). Statistical Evidence for the Relation Between Citrate and Carbonate in Human Cortical Bone. Calcif Tissue Int., 37, 363–366.

19. Kodde, A. K. (2003). Development of a Bioreactor for Bone Tissue Engineering.

20. Lakes, R. (1993). Materials with Structural Hierarchy. Nature, 361, 511–515. doi:10.1038/361511a0

21. Leonards, J. R., & Free, A. H. (1944). The Citrate Content of the Skeleton as Influenced by Acid-Producing and Base-Producing Salts. J. Biol. Chem., 155, 503–506.

22. Lopez-Macipe, A., Gomez-Morales, J., & Rodriguez-Clemente, R. (1998). The Role of pH in the Adsorption of Citrate Ions on Hydroxyapatite. Journal of Colloid and Interface Science, 120(200), 114–120.

23. Pate, F. D., & Norrish, K. (1989). Ionic exchange between soil solution and bone : toward a predictive model. Applied Geochemistry, 4, 303–316.

24. Pörtner, R., Nagel-heyer, S., Goepfert, C., Adamietz, P., Meenen, N. M. (2005). Bioreactor Design for Tissue Engineering. Journal of Bioscience and Bioengineering, 100(3), 235–245. doi:10.1263/jbb.100.235

25. Saxberg, B. E. H., & Kowalski, R. (1979). Generalized Standard Addition Method. Analytical Chemistry, 51(7), 1031–1038.

26. Schwarcz, H. P., Agur, K., & Jantz, L. M. (2010). A new method for determination of postmortem interval: citrate content of bone. Journal of Forensic Sciences, 55(6), 1516–22. doi:10.1111/j.1556-4029.2010.01511.x

27. Taylor, P. J. (2005). Matrix effects : The Achilles heel of quantitative high-performance liquid chromatography – electrospray – tandem mass spectrometry. Clinical Biochemistry, 38, 328–334. doi:10.1016/j.clinbiochem.2004.11.007

28. Tibbett, M., & Carter, D. O. (Eds.). (2008). Soil Analysis in Forensic Taphonomy: Chemical and Biological Effects of Buried Human Remains (pp. 203–270). CRC Press, Inc.

29. Ubelaker, D. H. (1997). Forensic Taphonomy: The Postmortem Fate of Human Remains. In Taphonomic Applications in Forensic Anthropology (1st ed., pp. 77–90). CRC Press, Inc.

30. USDA, & NRCS. (2010). Keys to Soil Taxonomy. (S. S. Staff, Ed.) (11th ed.). United States Department of Agriculture: Natural Resources Conservation Service.

31. Vass, A. A. (2011). The elusive universal post-mortem interval formula. Forensic Science International, 204, 34–40. doi:10.1016/j.forsciint.2010.04.052

32. Vass, A. A., Ph. D., Bass, W. M., Wolt, J. D., Foss, J. E., & Ammons, J. T. (1992). Time Since Death Determinations of Human Cadavers Using Soil Solution. Forensic Sciences, 37(5), 1236–1253.

33. Weiner, S., & Wagner, H. D. (1998). THE MATERIAL BONE: Structure-Mechanical Function Relations. Annual Review of Materials Science, 28(1), 271– 298.doi:10.1146/annurev.matsci.28.1.271

34. White, E. M., & Hannus, L. A. (1983). Chemical Weathering of Bone in Archaeological Soils. American Antiquity, 48(2), 316–322.

35. Yang, H., Hao, L., Du, C., & Wang, Y. (2013). RSC Advances hydroxyapatite in the presence of citrate. RSC Adv., 3(002), 23184–23189. doi:10.1039/c3ra44839h